A new role for Filamin A as a regulator of Runx2 function

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Abstract

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Filamin A is a well-characterised cytoskeletal protein which regulates cell shape and migration by cross-linking with actin. Filamin A mutations cause a number of human developmental disorders, many of which exhibit skeletal dysplasia. However, the molecular mechanisms by which Filamin A affects skeletal development are unknown. The transcription factor Runx2 is a master regulator of osteoblast and chondrocyte differentiation. Data presented in this thesis show that Filamin A forms a complex with Runx2 in osteoblastic cell lines. Moreover, it is demonstrated that Filamin A is present in the nucleus in several cell lines, including those of osteoblastic origin. The data presented show that the Filamin A/Runx2 complex suppresses the expression of the gene encoding the matrix-degrading enzyme, matrix metalloproteinase-13 (MMP-13), which is an important osteoblastic differentiation marker. ChIP assays were employed to demonstrate that endogenously expressed Filamin A associates with the promoter of the *MMP-13* gene. In addition, Filamin A is not only located in the nucleus but also in the nucleolus, an important nuclear compartment involved in ribosomal RNA (rRNA) transcription. Ribosomal DNA promoter-driven reporter assays, Filamin A-knockdown experiments and exogenous Filamin A transfections demonstrated that Filamin A and Runx2 can repress ribosomal gene expression activity. Importantly, Filamin A is recruited to the human ribosomal DNA promoter, suggesting its direct involvement in the regulation of rRNA transcription. These findings reveal a novel role of Filamin A in the direct regulation of ribosomal gene expression. Finally, by using microarray technology, changes in gene expression were identified when Filamin A was downregulated. Some of the differentially expressed genes were known orchestrators of bone development. The data presented in this thesis strengthen the link between Filamin A and bone development and provide a molecular rationale for how Filamin A, acting as a regulator of gene expression, might influence osteoblastic differentiation.

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Abbreviations

AMD	Actinomycin D
AML	Acute Myeloid Leukaemia
ATCC	American Type Culture Collection
AR	Androgen receptor
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
BSP	Bone sialoprotein
CBFβ	Core binding factor beta
CBP	CREB binding protein
CCD	Cleidocranial dysplasia
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CMV	Cytomegalovirus
CREB	CCAAT enhancer binding protein
DAVID	Database for annotation, visualization and integrated discovery
DAPI	4,6-diamidino-2-phenylindole
DFC	Dense fibrillar component
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide-5'-triphosphate
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra-acetic acid
EGTA	Ethylene Glycol-bis (β-aminoethylether) -N, N, N', N' Tetraacetic acid
ER	Estrogen receptor
FBS	Foetal bovine serum

FGF	Fibroblast growth factor
FC	Fibrillar centre
FlnA	Filamin A
GAPDH	Glyceraldehyde-3-phosphate dehydogenase
GFP	Green fluorescent protein
GR	Glucocorticoid receptor
GC	Granular component
HATs	Histone acetyl-transferases
HEPES	N-2-hydroxyethylpiperazine -N'-2-ethanesulfonic acid
HDACs	Histone deacetylases
IgG	Immunoglobulin G
ICA	Intensity correlation analysis
kDa	KiloDalton
LB	Luria broth
MMP-13	Matrix metalloproteinase-13
MAP	Mitogen activated protein
MEM	Modified Eagle's medium
mRNA	Messenger RNA
NFκB	Nuclear factor κB
NOPdb	Nucleolar Proteome Database
NoLS	Nucleolar Localisation Signal
NLS	Nuclear localisation signal
NPM	Nucleophosmin
NMTS	Nuclear Matrix Targeting Signal
OPN	Osteopontin
OPG	Osteoprotegerin
OSC	Osteocalcin

ON	Osteonectin
OSE-2	Osteocalcin specific element 2
PAGE	Polyacrylamide Gel Electrophoresis
Py	Pyrimidine
PBS	Phosphate buffer saline
PMSF	Phenylmethylsulphonyl fluoride
PTH	Parathyroid hormone
RANK	receptor activator of NF-kB
RANKL	Receptor activator of NF-kB ligand
RHD	Runt homology domain
rDNAp	Ribosomal DNA promoter reporter plasmid
RNA	Ribonucleic acid
RT-qPCR	Quantitave Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SD	Standard deviaton
siRNA	small interference RNA
STAT	Signal transduction and activator of transcription
TAE	Tris acetate / EDTA electophoresis buffer
TAF	TBP-associated factor
TBE	Tris borate / EDTA electophoresis buffer
TBS	Tris buffered saline
TE	Tris EDTA
TEMED	N, N, N', N' - tetramethylethyl-enediamine
TGFβ	Transforming growth factor β
TNFα	Tumour necrosis factor α
UBF-1	Upstream binding factor 1

Publications and conferences derived from this work.

Publications

a) Cesar Lopez-Camacho*, Wensheng Deng*, Jen-Yang Tang, Daniel Mendoza Villanueva, Apolinar Maya-Mendoza, Dean A. Jackson and Paul Shore **The cytoskeletal protein, Filamin A, is a nucleolar protein that suppresses ribosomal RNA gene transcription.** Manuscript in preparation.

b) Cesar Lopez-Camacho, Jen-Yang Tang, Wensheng Deng, Claire K. Inman, Daniel Mendoza-Villanueva and Paul Shore. **The genetic regulation of matrix metalloproteinase-13 (MMP-13) by Runx2 is dependant on Filamin A interaction.** Manuscript in preparation.

Oral Presentations

a) Runx transcription factors conference. Hiroshima, Japan. July 2010.
b) Symposium of Mexican Students and Studies in UK. University of Manchester. July, 2010
c) Symposium of Mexican Students and Studies in UK. Imperial College of London. June 2008
d) Workshop of Molecular Cancer Studies, University of Manchester. March, 2009

Poster Presentations

a) Runx transcription factors in development and disease, University of Oxford, United Kingdom, August 2009.

1.0 Introduction

1.1 The skeleton

The skeleton, primarily composed of osseous tissue and cartilage, provides support to the entire body by forming a structural design where soft tissue and vital organ systems attach. The hardness of bones creates a protective barrier for organs; for example, the spinal cord is protected by the vertebrae, the rib cage protects the lungs and the heart and the skull protects the brain. The skeleton is essential for locomotion and is where the muscles are attached, thus enabling mobility. Chemically, bones are made from calcium, phosphate and a fibrous protein. Bones are reservoirs for lipids and minerals which have essential roles in maintaining homeostasis (Dorozhkin & Epple, 2002). Another function of the skeleton is to harbour the bone marrow, which functions to produce blood cells (Del Fattore et al, 2010). Given the biological significance of the skeleton, it is important to study the molecular mechanisms which govern bone formation and bone homeostasis. One important player in bone formation is the transcription factor Runx2. Runx2 is a specific DNA binding protein that regulates the expression of a large group of molecules involved in the generation and stability of the bone. This thesis describes a novel interaction between Runx2 and the cytoskeletal protein Filamin A. The role of this interaction as a novel mechanism to regulate bone gene expression is investigated.

1.2 Skeletal development

In order to produce skeletal tissue, a well-orchestrated process which involves the migration and differentiation of a number of cell-types must occur. Skeletal formation requires that a specific type of stem cells, governed by several transcription factors, are committed to display osteoblastic phenotype (Gordeladze et al, 2009). This occurs via two different mechanisms: endochondral ossification and intramembranous ossification. Endochondral ossification is the process of bone formation starting from cartilage and is the course by which most of the skeleton forms (Ducy & Karsenty, 1998). The first step is characterised by the migration of undifferentiated mesenchymal cells into areas destined to become bone. Mesenchymal cells organise and are the template to form bone. Cells within this organisation begin to express collagen type I and type IIa and start to differentiate into chondrocytes that will secrete extracellular matrix to form cartilage (Ducy & Karsenty, 1998). The chondrocytes mature, become hypertrophic and commence to secrete type X collagen and mineralize the surrounding matrix. Vascular invasion from the perichondrium and apoptosis of hypertrophic chondrocytes make way for mesenchymal cells that differentiate into osteoblasts. Osteoblasts form bone by secreting proteins and structural elements that form a collagen and mineralized extracellular matrix (Karsenty, 2001). In contrast to endochondral ossification, intramembranous ossification does not require cartilage. Mesenchymal condensations vascularise and precursor cells differentiate directly into osteoblasts to form the mineralized bone (Ducy & Karsenty, 1998). Intramembranous ossification gives rise to the flat bones that comprise the cranium and medial clavicles and it is an essential process during the natural healing of fractures.

1.3 Cells in bone

Bone tissue contains mainly five different cell-types; osteogenic cells, osteoblasts, osteocytes, osteoclasts and chondrocytes (Kartsogiannis & Ng, 2004). In general, there are cells that are responsible for the response of the body to trauma or fracture, and those which secrete components which bones are made of. There are cells that make bone and cells that degrade bone. Thus, these two cell-types must be in equilibrium in order to maintain bone homeostasis. Osteoblasts are responsible for bone formation and deposit bone by secreting osteid, a mixture of bone matrix proteins. Osteoblasts are derived from multipotent mesenchymal stem cells and their differentiation is controlled by several growth factors, hormones and transcription factors (Jensen et al, 2010). Two transcription factors, Runx2 and osterix are absolutely essential for osteoblast differentiation (Ducy & Karsenty, 1998; Kobayashi & Kronenberg, 2005). The major protein secreted by osteoblasts is collagen type 1. Osteoblasts also secrete non-collagenous proteins: osteocalcin, osteonectin and alkaline phosphatase which are important in regulating bone formation. Osteoblasts direct the mineralization of bone matrix by fluctuating concentrations of calcium and phosphate ions and induce formation of hydroxyapatite deposits.

Osteocytes are derived from osteoblasts and constitute around 95% of all bone cells. Osteocytes function to maintain bone matrix, they have a characteristic morphology that resembles neurons and occupy a space termed lacunae. These interconnected cells create an osteocytic network that forms a functional syncytium which is needed for communication and transmission of mechano-sensory signals via changes in interstitial fluid movement produced by mechanical forces. This arrangement permits osteocytes to

act upon any signal and thus secrete secondary messengers, activating transcription and altering gene expression (Dallas & Bonewald, 2010).

Osteoclasts are multinucleated cells that derive from hematopoietic progenitors in the bone marrow and mediate the resorption of mineralized bone matrix and its differentiation is directed by cell-cell contact with either bone marrow stromal cells or osteoblasts (Kobayashi et al, 2009). The receptor activator of NF-kB ligand (RANKL), a transmembrane protein expressed in osteoblasts, binds to activator of NF-kB (RANK) which is expressed on the surface of osteoclast precursors. Formation of RANKL-RANK complex stimulates differentiation and activation of osteoclasts (Suda et al, 1999). Osteoprotegerin (OPG), secreted by stromal cells and osteoblasts acts as an antagonist molecule by binding to RANK and thus preventing RANKL-induced differentiation.

Chondrocytes are differentiated cells found in cartilage, a flexible connective tissue found in many areas in the body. Cartilage serves as a template for future bones in the developing embryo and forms the growth plate in long bones. Once differentiated, chondrocytes function to synthesize and maintain the extracellular matrix of cartilage as well as undergoing proliferation, hypertrophy and secreting matrix in growth plates to promote longitudinal bone growth (Shum & Nuckolls, 2002).

1.4 The importance of gene regulation during skeletal development

The formation of bone tissue depends on the proliferation and differentiation of specific cell-types. For example, in bone development, osteoblasts are very important in order to ensure correct mineralization; and osteoclasts are required to maintain the equilibrium between bone formation and bone degradation. Therefore, because transcription factors

control cell fate, a correct regulation of the genes involved in this process is essential. A group of transcription factors play important roles in the regulation of gene expression and thus promote bone formation (Fig. 1.0). A failure in the regulation of gene expression during bone formation can lead to skeletal abnormalities during development and adulthood. This can be the case for the transcription factor Runx2 which is a master regulator in bone formation. In this thesis, a novel mechanism of regulation of Runx2 in osteoblastic cells is proposed. In consequence, an overview of the transcriptional regulation in osteoblasts will be provided.

1.5 The transcriptional control of osteoblastogenesis.

In endochondral bone formation, in order to produce a mature osteoblast from a mesenchymal cell, a set of transcription factors regulates the expression of certain genes (Fig. 1.0). Here, the role of the key transcription factors involved in the formation of osteoblasts is briefly outlined. First, mesenchymal cells, by the influence of the transcription factor Sox-9, transform into skeletal precursor cells. These precursors in turn will differentiate into osteoblasts or chondrocytes. In this regard, Sox-9 plays dual roles; it inhibits osteoblast differentiation but also induces chondrocyte maturation (Akiyama, 2008) (Fig. 1.0). The fate of the skeletal cells will depend on the expression and function of several transcription factors. For example, the skeletal precursors destined to be part of the osteoblastic lineage express the transcription factors Dlx5 and Msx2 which promote osteoblast proliferation and differentiation (Shirakabe et al, 2001). Dlx5 and Msx2 regulate the expression of the transcription factor Runx2, a master regulator of bone formation (Lee et al, 2005; Samee et al, 2008; Shirakabe et al, 2001). The function of Runx2 is also regulated by Runx2-binding proteins that further regulate

its activity. Runx2 transcriptionaly regulates the expression of the transcription factor Osterix/Sp7. Osterix is critically important to fully commit a Runx2-expressing osteoblastic precursor to become an osteoblast and ensures the differentiation into osteoblasts instead of chondrocytes (Maruyama & Komori, 2007; Matsubara et al, 2008). Immature osteoblasts are characterised by the expression of the transcription factor Fra1 which controls the expression of various matrix genes, thereby regulating matrix production and providing functional environment for mature osteoblasts (Eferl et al, 2004). Once the cell fate has been programmed, the immature osteoblasts differentiate into mature osteoblasts (Fig. 1.0). In this process Msx2 is downregulated as well as Runx2 (Shirakabe et al, 2001). At this stage Msx2 inhibits Runx2 activity and osteoblast gene expression (Liu et al, 2001). Also, at this stage, the transcription factors Sp3 and Atf4 are required during late osteoblastogenesis (Gollner et al, 2001; Yang & Karsenty, 2004).

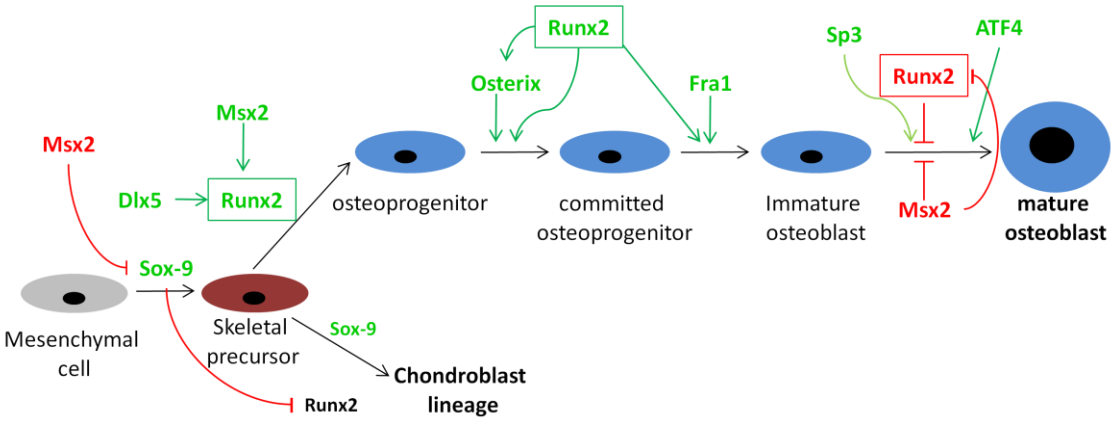


Fig. 1.0 Schematic representation of the activities and regulation of the key transcription factors during differentiation and maturation of osteoblasts. Positive influences are indicated by green arrows and positive factors are labelled green. Negative influences are indicated by blunted red lines and negative acting factors are labelled red [Modified from (Hartmann, 2009)]

1.6 Runx family of transcription factors

1.6.1 The Runt domain

The transcriptional activity of Runx2 is essential to promote correct bone formation and osteoblast maturation. Runx2 belongs to the Runx family of transcription factors which have in common a Runt domain. The Runt domain, located towards the amino-terminal end, is composed of 128 amino acids (Ito, 2004). This domain was called Runt because it was first identified in the *runt* gene in *Drosophila melanogaster*, which has a role in sex determination, neurogenesis and segmentation (Blyth et al, 2005). The Runt domain is highly conserved between species ranging from invertebrates to vertebrates (Rennert et al, 2003). A large body of evidence has demonstrated that Runx proteins are key mediators in several pathways of development (Coffman, 2003). In mammals, the Runt domain is very similar between Runx1, Runx2 and Runx3 (van Wijnen et al, 2004). However, these proteins are capable of activating different genes in different cell types. Those activated genes are mainly involved in development, proliferation and differentiation (Nimmo & Woollard, 2007). The Runt domain can bind to the specific recognition sequence PyGPyGGTPy (Py = pyrimidine), (Kamachi et al, 1990). The three dimensional structure of the Runt domain has been determined by Nuclear magnetic resonance and crystallography (Bartfeld et al, 2002; Bravo et al, 2001; Zhang et al, 2003). It contains ten anti-parallel and two parallel beta-sheet strands (Ito, 2004) (Fig. 1.1). The beta strands form an s-type immunoglobulin fold and are connected by loops which mediate DNA binding. Also, it has been shown that the C-terminal tail is necessary to make specific DNA contacts (Zhang et al, 2003).

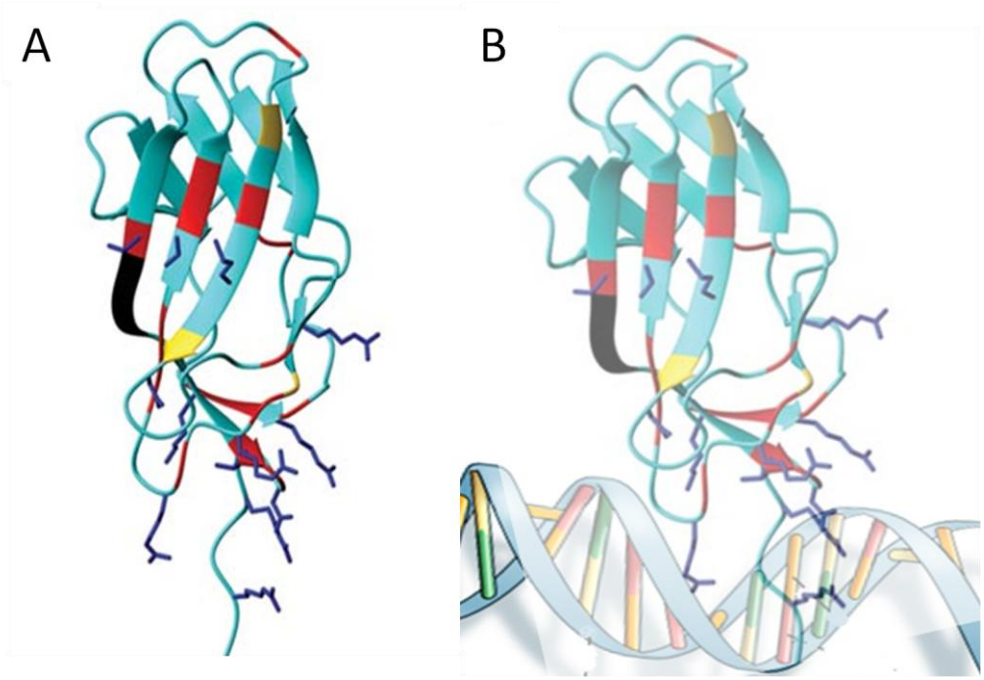


Fig. 1.1 Schematic representation of the Runt domain. A) A ribbon diagram showing the solution structure of the Runt domain. The Runt domain is composed of 6 anti parallel and 4 parallel β-strands. The amino acids located between those β-strands form several loops which are very important in recognizing and binding to the specific sequence of DNA. B) A ribbon diagram showing the interaction between the Runt1 domain and the DNA [Adapted from (Cohen, 2009)].

Despite the fact that the Runt domain can specifically bind to DNA, this binding can be increased when CBFβ, a 22 kDa protein, binds to the Runt domain. However, CBFβ has not been reported to bind directly to DNA sequences (Nagata et al, 1999). The binding of CBFβ, which is the heterodimerisation partner of all Runx transcription factors enhances its ability to bind to DNA and can also protect the Runx protein from ubiquitin-proteasome degradation (Huang et al, 2001). CBFβ does not bind directly to DNA but on association with the Runt domain it enhances the affinity of Runx proteins to DNA (Bartfeld et al, 2002; Nagata et al, 1999). The DNA-Runt conformation stabilises in the presence of CBFβ by hydrogen bonds and hydrophobic interactions. The three-dimensional structure of the Runt domain/CBFβ complex associated with DNA (Fig. 1.2) has been determined (Bartfeld et al, 2002; Bravo et al, 2001). By doing this it has been shown that the Runt domain suffers a conformation change when binding to DNA which is known as the S-switch.

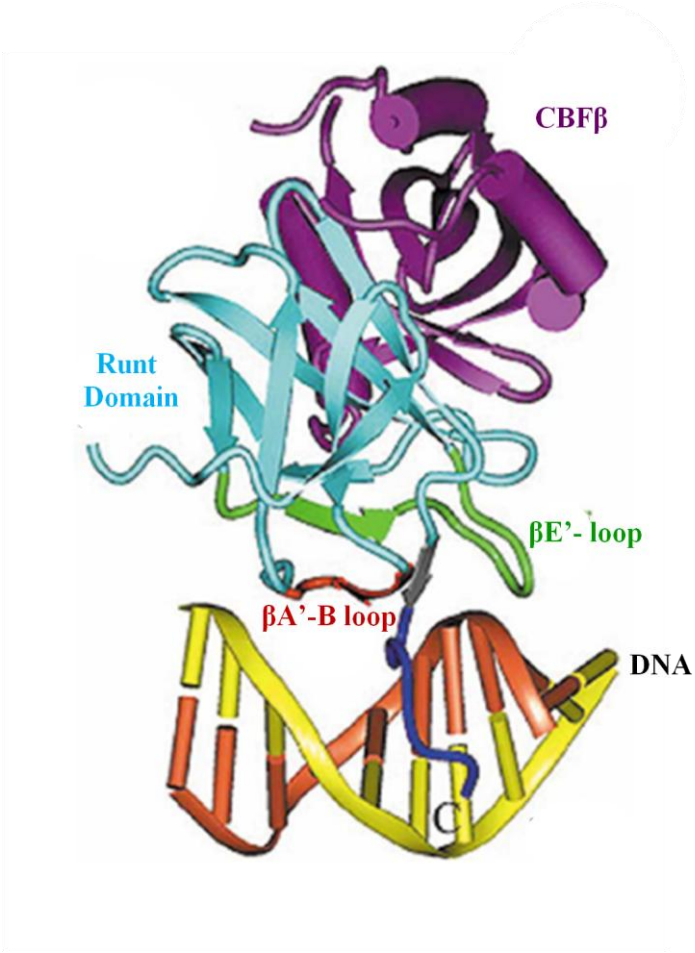


Fig. 1.2 Structure of the Runx1 Runt domain-CBFβ-DNA complex. A ribbon diagram of the Runt domain-CBFβ heterodimer and its association with the DNA. The Runt domain is shown in cyan and the contacting CBFβ in purple. Runt domain makes contact with DNA through its C-terminal loop and the βA'-B loop. This interaction involves hydrogen bonds; however, CBFβ enhances this binding by eliciting a conformation change in the Runt domain that makes the contact with the DNA stronger. [Adapted from (Bartfeld et al, 2002; Bravo et al, 2001)].

1.7 Human Runt Family of transcription factors

The human genome contains three genes encoding *Runx1*, *Runx2* and *Runx3* (Fig. 1.3). The Runt Domain, the Nuclear Matrix Targeting Signal (NMTS), the Nuclear Localisation Signal (NLS) and the carboxy-terminal VWRPY motif are conserved in all three human Runx proteins (Wheeler et al, 2000). These transcription factors have both activation and inhibitory domains. *RUNX* genes can be expressed from two different promoters giving rise to two isoforms with different amino-terminal amino acids (MASNS and MRIPV) (Wheeler et al, 2000). In the following paragraphs, the biological features of the Runx proteins will be summarized.

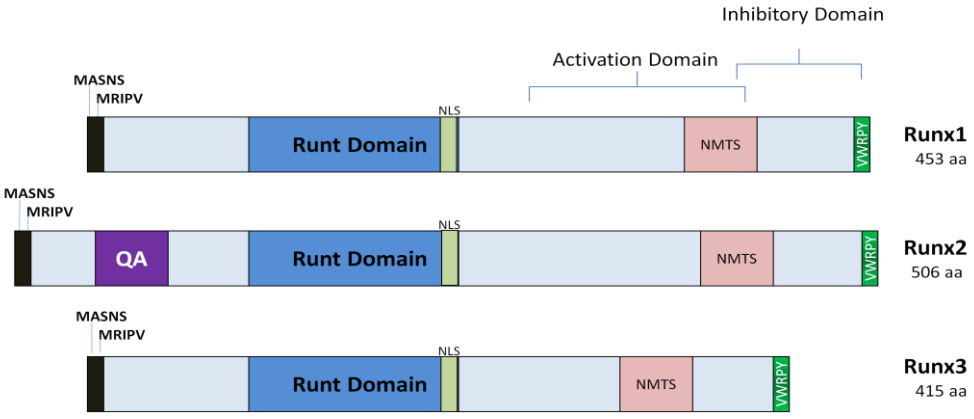


Fig. 1.3 The structure of Runx proteins. Diagrammatic representation of Runx1, Runx2 and Runx3. Conserved Runt domain (blue) is 128 amino acids length and it is responsible of the recognition and binding to DNA sequence. Nuclear Matrix Targeting Signal (NMTS), in pink and Nuclear Localisation Signal (NLS), in light green are found in all Runx proteins. The C-terminal contains the sequence VWRPY (green), which can bind to Runx co-repressors. MASNS and MRIPV aminoacids represent two different isoforms.

1.8 The biological function of Runx family of transcription factors.

1.8.1 The transcription factor Runx1

RUNX1 gene, located on the chromosome 21 in humans an in chromosome 16 in mouse is also known as AML1, Cbfa2, or Pebp2α, encodes a protein of 453 amino acids and has 69% identity with the Drosophila gene runt (Ito, 2004). It has been shown that Runx1 is not crucial for morphogenesis but it is important for haematopoietic cell formation (Bee et al, 2010; de Bruijn & Speck, 2004). When *Runx1* is ablated, mice at the embryonic day 12.5 die because the lack of fetal liver haematopoiesis (Okuda et al, 1996; Wang et al, 1996). Since *Runx1* ablation is lethal in mice, its function on skeletal development is not well known. However, it has been demonstrated that *Runx1* is crutial to form the sternum even in precense of Runx2 (Kimura et al, 2010).

1.8.1.1 Runx1 and disease

Leukaemia is a cancer which disrupts the cell proliferation control of blood cells. Acute leukaemia is the most common leukaemia presented in patients and is characterised by the rapid proliferation of immature blood cells. The majority of the leukaemias are caused by chromosomal translocations in which a gene can rearrange its sequence with a non related gene, thus creating a fused protein that will impair the biological role of the proteins involved. It has been shown that Runx1 is a recurrent target of chromosomal translocation in human leukaemias. Nowadays, 23 translocations involving *RUNX1* have been described (Dai et al, 2007). The biological consequence of chromosomal translocations can be the loss of protein functionality but also the gain of function (Yamagata et al, 2005).

1.8.2 The transcription factor Runx3

The *RUNX3* gene, also known as AML2, Cbfa3, or Pebp2αC, is located on the short arm of chromosome 1 (1p36.1) in humans and on chromosome 4 in mouse. It transcribes into 5 different mRNA isoforms produced by alternative splicing in humans (Puig-Kroger & Corbi, 2006). Runx3 has been found to be expressed in myeloid cells, the gastric epithelial layer (Puig-Kroger & Corbi, 2006) and in he tyrosine kinase receptor C (TrkC) proprioceptive neurons (Levanon et al, 2002). However, Runx3 expression in the gastrointestinal tract is controversial. Two groups have produced Runx3 knockout mice (Levanon et al, 2002; Li et al, 2002) in which two comparable defects were identified, one defect involved neurogenesis and the other thymopoiesis. In addition, a stomach defect in gastric cancer was observed in one of the mutant strains, but not in the other (Levanon et al, 2002). This observation strongly suggests that loss of Runx3 is not necessarily associated with gastric neoplasia and further studies are trying to conceal the discrepancies observed.

1.8.2.1 Runx3 and disease

Runx3 has been proposed to have a role in cancer. It has been shown that *Runx3* knockout mice can present high proliferation rates and lack of apoptosis, leading to hyperplasia in the gastric tract. In contrast, transfected wild type Runx3 in gastric carcinoma cells reduced tumour formation (Li et al, 2002). The above data is supported by the fact that nearly 60% of human gastric cancer cells do not produce Runx3 (Anglin & Passaniti, 2004). Taken together, those findings suggest that lack of Runx3 is strongly related to human gastric cancer.

1.9 The transcription factor Runx2

1.9.1 Biological significance of Runx2

Runx2 regulates a vast number of genes that are necessary to activate bone and cartilage formation (Komori et al, 1997). Runx2 as an osteoblast specific transcription factor was discovered through the analysis of the osteocalcin promoter. In 1995, two different *cis-acting* elements which controlled the expression of the osteocalcin promoter were identified in rodents. One of them, the osteocalcin specific element 2 (OSE-2) was highly active in mature osteoblasts and its nucleotide sequence was similar to the DNA-binding site of the transcription factor PEBP2α/AML1/Runx1 (Ducy & Karsenty, 1995; Geoffroy et al, 1995). Runx2 was able to strongly induce the promoter activity of a luciferase reporter construct which consisted of multimers of OSE-2 (Ducy et al, 1997). To further characterise the function of Runx2, its expression was evaluated during development by the generation of a *Runx2*- deficient mouse (Komori et al, 1997; Otto et al, 1997; Stein et al, 2004). Homozygous mutants died of respiratory failure shortly after birth. Analysis of their skeletons revealed an absence of osteoblasts and bone (Fig. 1.4) and heterozygous mice showed specific skeletal abnormalities that are characteristic of the human heritable skeletal disorder, cleidocranial dysplasia (CCD). Analysis of embryonic Runx2 expression using a lacZ reporter gene revealed strong expression at sites of bone formation (Otto et al, 1997). Further analysis of the skeleton revealed small populations of immature osteoblasts and osteoclasts in the perichondrial region where osteogenesis occurs (Komori et al, 1997; Otto et al, 1997). The presence of OSE2-like elements in the promoters of some osteoblast specific genes indicated that Runx2 regulated their expression. Over-expression of Runx2 in osteoblast and non-

osteoblast cells lines caused the expression of several osteoblast specific genes (Ducy et al, 1997). Thus, Runx2 is considered to be primarily an osteoblast-specific transcription factor essential for osteoblastic differentiation.

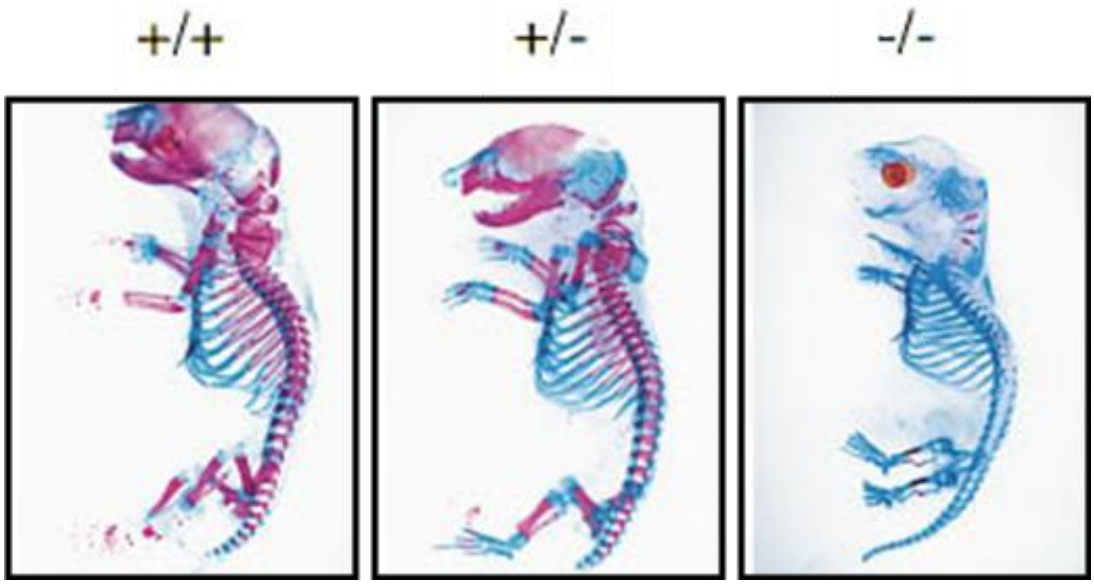


Fig. 1.4 Lack of ossification in *Runx2* null mice. Mice skeletons from wild-type, heterozygous and homozygous genotype of Runx2. Specimens were double stained with Alizarin to demonstrate calcification (red) and Alcian to demonstrate cartilage formation (blue). It can be observed the lack of ossification in the Runx2 -/- mutant mouse (Komori et al, 1997).

1.9.2 Runx2 and skeletal disease.

1.9.2.1 Cleidocranial Dysplasia

Cleidocranial Dysplasia (CCD) is a skeletal disorder with autosomal dominant inheritance. The clinical manifestations of CCD (Fig. 1.5) are defective clavicles, delayed closure of cranial fontanels, short stature, dental malformation and supernumerary teeth (Mundlos, 1999). The locus of CCD is located in the chromosome 6p21, where the *RUNX2* gene is located. The majority of patients who suffer CCD have a mutation in *RUNX2* gene (Schroeder et al, 2005). A closer look at the *RUNX2* gene locus in CCD families revealed that the disease phenotype is due to deletion, insertion and nonsense mutations that lead to premature stop codons in the Runt domain or in the C-terminal transactivation domain (Mundlos, 1999). Other causative alterations include missense mutations that abolish the DNA binding activity of Runx2, mutations in the SMAD interacting domain, frame shift mutations, disruption of splice donor sites and mutations within the promoter (Kim et al, 2006; Lee et al, 1997; Napierala et al, 2005; Quack et al, 1999; Zhang et al, 2000). Careful analysis of genotypes and corresponding phenotypes failed to indicate any correlation between the types of mutations and the clinical severity of the disorder. The absence of obvious differences in phenotype between patients suggested that the disease is generally caused by *RUNX2* haploinsufficiency rather than specific changes in function (Quack et al, 1999).

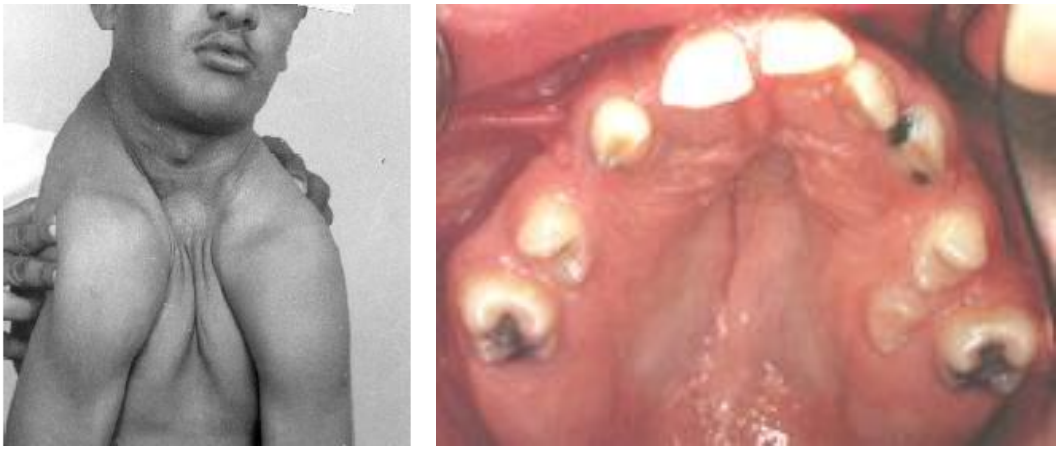


Fig. 1.5 The clinical manifestations of Cleidocranial Dysplasia (CCD). *RUNX2* gene mutations can lead to CCD which affects principally the normal osteogenic process in humans. In CCD, shoulders in a patient with clavicular hypoplasia may be brought to the midline (right). CCD also causes dental malformations and teeth located on or around the palate (Left). Figure taken from www.pediatriconcall.com

1.10 Structural and functional domains of Runx2

The *RUNX2* gene, located on chromosome 6, is also known as AML3, Cbfa1, OSF-2 and Pebp2αA (Ogawa et al, 1993). This gene encodes a 513 amino acids protein which contains the Runt domain in the position 103 to 217 (see Fig 1.3). In humans, the *RUNX2* gene transcribes into 2 different mRNA molecules produced by alternative splicing. Those isoforms have different sequences in the amino terminal. The isoform II (Runx2-II) starts with the amino acid sequence MASNS and is found only in osseous cells and isoform I (Runx2-I) starts with MRIPV and is commonly found in osteoblast as well as in spleen and T cells (Banerjee et al, 2001; Prince et al, 2001). The Runx2 isoforms share the same functional domains (Stock & Otto, 2005) which include the glutamine/alanine rich (Q/A) tract, the Runt DNA binding domain, the nuclear localisation signal (NLS), the PST domain, the nuclear matrix targeting signal (NMTS) and the C-terminal pentapeptide VWRPY (See Fig. 1.3). Domains unique to Runx2 participate in the transactivation function: activation domain 1 (AD1) consists of the first 19 amino acids of Runx2-II and its deletion resulted in a 4-fold decrease in transactivation function (Thirunavukkarasu et al, 1998). The second transactivation domain (AD2) was determined to be the Q/A sequence and its deletion caused a 75% decrease in Runx2 transcriptional activity. Activation domain three (AD3) was found in the PST region of Runx2. Complete removal of PST region resulted in a four to five fold decrease in the Runx2 transactivation function (Thirunavukkarasu et al, 1998). A characteristic of human and mouse Runx2 are the successive polyglutamine and polyalanine amino acids (Q/A) domain which plays a significant role in transactivation function (Thirunavukkarasu et al, 1998). The Nuclear Localisation Signal (NLS) is also

present in Runx2 protein. Removal of the 9 amino acids of the NLS results in cytoplasmic accumulation of Runx2. Furthermore, mutant Runx2 lacking the NLS was unable to transactivate the OSE2-luciferase promoter, demonstrating that the NLS is essential for transactivation function (Thirunavukkarasu et al, 1998). Once Runx2 is transported into the nucleus, it is further localised to the nuclear matrix by virtue of a nuclear matrix targeting signal (NMTS). The NMTS of Runx2 consists of approximately 38 amino acids and located in the C-terminal region (Zaidi et al, 2001; Zeng et al, 1997). NMTS targets Runx2 to specific subnuclear domains contributing to the effective transactivation of the osteocalcin promoter (Zaidi et al, 2001). The C-terminal end of Runx2 interacts with proteins which function to modulate its activity by either enhancing activation or by promoting repression of target genes. The pentapeptide sequence, VWRPY, which is present in all Runx proteins is localised at the very end of the C-terminus and is involved in the interaction with members of the groucho family of co-repressors (Aronson et al, 1997). In Runx2, deletion of VWRPY increased transactivation, suggesting that this region had repressor function. In addition, further C-terminal removal resulted in an increase of transactivation, indicating that the inhibitory domain was about 154 amino acids long (Thirunavukkarasu et al, 1998). Furthermore, it has been shown that over expression of the Groucho family members TLE1 and TLE2 repressed Runx2-induced osteocalcin promoter activity (Javed et al, 2000).

1.11 Modulation of Runx2 by co-regulatory proteins

Runx2 can activate or repress gene transcription. This dual function depends on the regulatory molecules that bind to either the activation or inhibitory domains (Wheeler et al, 2000). Activators or repressors can be expressed in different patterns and cell-types and this can explain how Runx2-dependent gene regulation can differ from one cell type to another.

1.11.1 Transcriptional co-activators of Runx2

In order to positively impact the transcription rate of Runx2-activated genes it is necessary that chromatin modification occurs and that the transcription machinery, formed by transcription factors, RNA polymerase II and activator molecules, is stabilised. As a representative example, Fig. 1.6 highlights some of the activators that bind to Runx2 and thus favouring transcription. The primary activation domain in Runx2 is a proline-serinethreonine (PST)-rich region that is distal to the nuclear localisation signal and immediately proximal to the NMTS domain (Kanno et al, 1998; Thirunavukkarasu et al, 1998). It contributes to interactions of Runx2 with p300, MOZ, and MORF (Pelletier et al, 2002; Sierra et al, 2003). The molecules P300/CBP (CREB binding protein) are histone acetyltransferase proteins that bind to Runx family proteins and in turn induce relaxations in chromatin structure by adding an acetyl group to the C-terminal of histones and thus exposing and facilitating the binding of transcription factors to DNA along with their regulatory molecules (Sierra et al, 2003). The amino-termini of Runx2 contain a polyglutamine and polyalanine (QA) domain that is not present in Runx1 or Runx3. Deletion studies have shown that Q/A region is important to activate Runx2. HES-1, a helix-loop-helix protein, also binds to Runx2 at the C-

terminus. However, this complex potentiates the transactivation function by preventing the binding of Groucho co-repressors family (McLarren et al, 2000). Interactions between Runx2 and other factors can be regulated via external stimuli, for example TGFβ and BMP can bind to receptors that in turn activate Smads which then interact with Runx2 (Ito & Miyazono, 2003). Many other factors have also been identified that can interact with Runx2 and result in an enhancement in the transcriptional activity. The co-activator TAZ can interact with Runx2, whilst interaction with the transcription factors Ets-1, Oct-1, AR/GR and AP1 can enhance transcription (Schroeder et al, 2005).

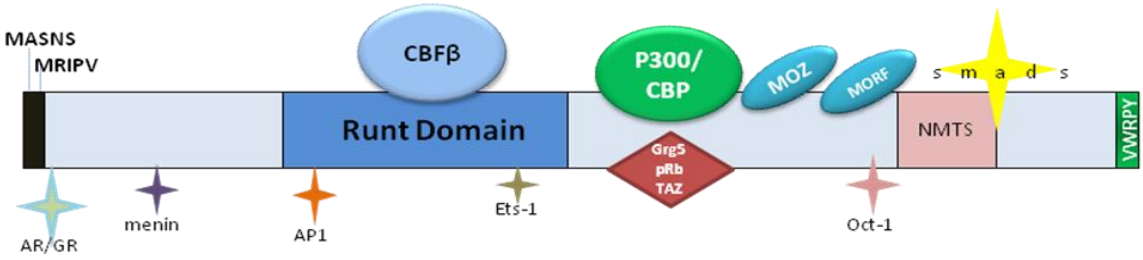


Fig. 1.6 The transcription factor Runx2 protein and the regulatory molecules that favour its transcriptional activity. Transcription factors (shown as stars) can bind to activation domains and induce Runx transcriptional activity. As an example, histone acetyltransferases as P300/CBP can complex to Runx2 and induce the relaxation of chromatin which will allow access of the basal transcription machinery to the promoter region. Other molecules such as MOZ and MORF also enhance the activation of the Runx transcription factor.

1.11.2 Transcriptional co-repressors of Runx2

Recent studies have identified several co-repressor molecules that bind to Runx2 proteins and regulate its transcriptional activity (Fig. 1.7) (Thirunavukkarasu et al, 1998). Repressors do not bind to DNA themselves and appear to act as follows: by preventing Runx2 from binding to the promoter sequence, by altering chromatin structure that cannot allow the access of transcription machinery complex, or by blocking co-activator complexes. Repressor proteins include histone deacetylases (HDACs), transducin-like enhancer of split proteins, mSin3a, and yes-associated protein (Westendorf, 2006) (Fig. 1.7). Runx2 contains a VWRPY motif. This region is responsible for recruiting the co-repressors TLEs which are the mammalian homologues of the Drosophila protein Groucho (Javed et al, 2000). Another co-repressor, mSin3a causes repression by the recruitment of HDACs (Lutterbach et al, 2000). mSin3a protects Runx2 from proteasome degradation but it also represses the expression of Runx2 target genes (Coffman, 2003). HDAC6 interacts with the C-terminal region of Runx2 and may deacetylate histones but also this association may alter the conformation of Runx2, deacetylate Runx2 or prevent the binding of co-activators to Runx2 (Westendorf et al, 2002). Another repression domain contained in the C-terminal of Runx2, mediates the binding to HDAC3 (Schroeder et al., 2004). Finally, the Runt domain of Runx2 interacts with the co repressor HDAC4 (Vega et al, 2004).

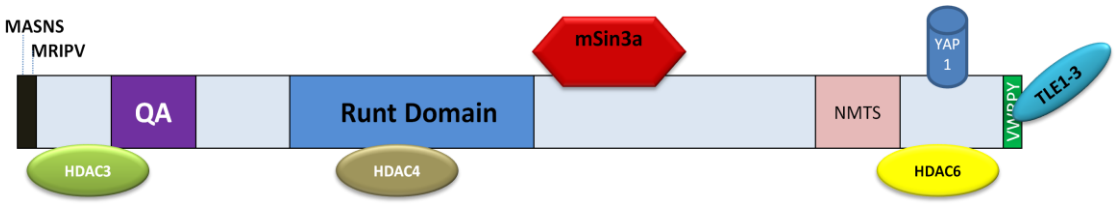


Fig. 1.7 The transcription factor Runx2 protein and the regulatory molecules that repress its transcriptional activity. As an example, histone deacetylases (HDACs) can complex to Runx2 and induce the chromatin to become more dense, which will not permit the access of the basal transcription machinery to the promoter region. Other molecules as mSin3a, TLE and YAP-1 also repress the activation of the Runx transcription factor.

1.12 Post-translational modifications of Runx2

The activity of Runx2 can be regulated by internal or external stimuli (Franceschi & Xiao, 2003). Fig. 1.8 summarizes how the activity of Runx2 can be regulated. The phosphorylation of Runx2 has been found to be a common modification that increases the transcriptional activation of Runx2. Type I collagen, which is present in the extracellular matrix can interact with β1 integrins on the cell surface. This interaction activates a focal adhesion kinase, which subsequently activates the ERK MAP kinase signalling pathway. Thus, the phosphorylation of Runx2 results in the upregulation of osteoblast specific genes that are required for differentiation, such as osteocalcin (Xiao et al, 2000). The activation of Runx2 by phosphorylation mediated by ERK does not only occur in response to signals from the extracellular matrix. Investigations have established that Fibroblast Growth Factor 2 (FGF-2) can stimulate Runx2 phosphorylation via the ERK pathway (Kim et al, 2003; Xiao et al, 2002). The

phosphorylation of Runx2 does not exclusively occur via ERK1/2 but can also be mediated by other kinases. It has been demonstrated that Protein Kinase A is capable of induce phosphorylation of Runx2. PKA can be activated in response to stimulation with parathyroid hormone (PTH), an important hormone involved in the regulation of calcium levels in the cell. PTH and Runx2 are jointly involved in the regulation of collagenase 3 in osteoblasts (Selvamurugan et al, 2004). Phosphorylation of Runx2 does not always result in an enhancement of its ability to activate transcription. The phosphorylation of Ser104 and Ser451 results in the repression of Runx2 transcriptional activation (Wee et al, 2002). Ser104 has also been implicated in Cleidocranial Dysplasia. Since this residue is mutated in some patients (Nakashima & de Crombrugghe, 2003), the phosphorylation or the mutation of Ser 104 may inhibit transcriptional activation by preventing Runx2 from binding with CBFβ (Wee et al, 2002). The activity of Runx2 may also be modified by acetyl and methyl-transferases. Runx2 is known to associate with the histone acetyl-transferases (HATs) p300 and MOZ. However, the role of HATs in the enhancement of expression of Runx2 target genes might not only be the acetylation of histones, but HATs might also acetylate Runx2 (Pelletier et al, 2002; Sierra et al, 2003).

The steroid hormone 1,25-(OH)2-vitamin D3 (VD3) regulates osteoblast differentiation by either activating or repressing transcription of numerous bone phenotypic genes. It has been shown that expression of Runx2 is downregulated by VD3 within 24 h, but not cells that lack a functional vitamin D receptor (VDR), indicating that VD3-mediated suppression of Runx2 activity is important to the control of genes involved during bone formation (Drissi et al, 2002). Conversely active vitamin D directly acts upon osteoclast

precursor cells and suppresses their differentiation in vitro (Takasu, 2008). Therefore vitamin D possess pleitropic effect in bone formation; it can increase bone volume by inhibiting accelerated bone resorption, but also it negatively regulates Runx2 expression.

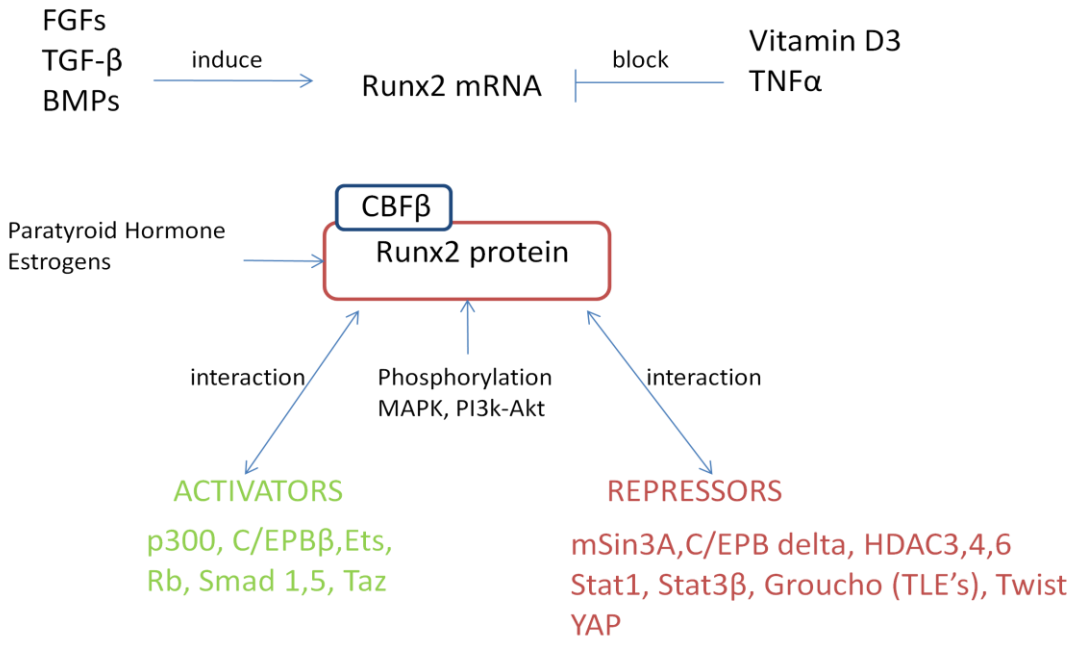


Fig. 1.8 The regulation of Runx2 transcription factor. Runx2/CBFβ binds to the specific DNA sequence via the runt domain to regulate gene expression. The extracellular signals as hormones and growth factors, the post-translational modifications as the acetylation and phosphorylation, and the interaction partners (activators or repressors) have important effects in the regulation of this transcription factor.

1.13 The Role of the cytoskeleton in the regulation of transcription

The cytoskeleton consists of a complex network of filamentous structures that includes intermediate filaments, microfilaments, actin filaments and microtubules (Janmey, 1998). The cytoskeleton maintains cell shape and stability, mediates cell movement and plays an important role in both intracellular trafficking and cellular division. In addition, recent evidence suggests that cytoskeletal proteins also have a role in regulating transcription factor function (Loy et al, 2003; Sasaki et al, 2001;Yoshida, 2005; Berry et al, 2005; Kim et al, 2007). For example, actin has been shown to regulate transcriptional machinery. Actin polymerization regulates the transcription factor Serum Response Factor (SRF) which controls growth-factor-regulated genes such as c-fos and cytoskeletal actin, and numerous muscle-specific genes. It has been shown that actin regulates the subcellular localisation of the myocardin-related SRF co activator MAL. MAL is redistributed from the cytoplasm to the nucleus in response to Rho-induced actin polymerization. Activation of the Rho-actin signalling pathway is necessary and sufficient to promote MAL nuclear accumulation, where it binds and activates SRF (Miralles et al, 2003). It has also been shown that CBFβ, the co-activator of Runx proteins associates with Filamin A, a component of the cytoskeleton that cross links actin filaments. In this case Filamin A retains CBFβ in the cytoplasm, thus preventing CBFβ from entering the nucleus to bind Runx1 (Yoshida, 2005).

1.14 Filamins

Filamins are large cytoplasmic proteins that crosslink F-actin to form a three-dimensional network of actin filaments. Filamins have been shown to interact with at

least 30 different cellular proteins (Popowicz, 2006) suggesting that they are important in a range of cellular processes.

1.14.1 Filamin and disease

Genetic evidence links Filamin as an essential protein for human development. Mutations in Filamin have been reported to cause developmental malformations and disease (Feng & Walsh, 2004) (Fig. 1.9). The first reported diseases involving Filamin A mutation was an X chromosome linked brain malformation called Perventricular Heterotopia (Lu & Sheen, 2005). This disease basically consists in the accumulation of neurons in the lateral ventricle as they were not able to migrate to the cerebral cortex. This miss-localisation of neurons causes Epilepsy in the second decade of life. Patients suffering Perventricular Heterotopia have abnormal mRNA splicing or early truncation of Filamin A protein. In rodents, mutations in Filamin A impede the neurons to leave the ventricular zone, originating brain malfunction (Feng & Walsh, 2004; Thomas et al, 1996). Filamin A malfunction causes a range of skeletal disorders including otopalatodigital syndrome types 1 and 2 (OPD1 and OPD2), frontometaphyseal dysplasia (FMD) and Melnick Needles syndrome (MNS), all of which exhibit skeletal dysplasia (Feng & Walsh, 2004). In mice with complete loss of Filamin A, abnormal epithelial and endothelial organisation and aberrant adherent junctions in developing blood vessels, heart, brain and other tissues are found (Feng et al, 2006). Missense mutations in Filamin A, however, lead to skeletal malformations (Feng et al, 2006; Robertson, 2005; Robertson et al, 2003). It is suggested that Filamin A plays important roles in cell-cell contacts and adherens junctions during organ development (Feng et al, 2006). In addition, mutations in the related protein Filamin B cause severe skeletal

abnormalities in spondylocarpotarsal syndrome (SCT), Larson syndrome and atelosteogenesis I and III (Dobbs et al, 2008; Farrington-Rock et al, 2006; Zheng et al, 2007). The expression of Filamin B is particularly high in human growth plate chondrocytes and in the developing vertebral bodies in the mouse (Krakow et al, 2004). Also, it has been shown that Filamin B mutations cause chondrocyte defects in skeletal development (Lu et al, 2007). Mice with disruption of both alleles of Filamin B have a high rate of embryonic death due to impaired development of microvasculature and skeleton (Farrington-Rock et al, 2008). Filamin B malfunction also results in skeletal defects in vertebral column and ribs (Zhou, 2007). Filamin A has some overlapping expression with Filamin B and could provide functional redundancy in some cell types (Sheen et al, 2002). However, despite being a clear genetic link between Filamin A and B in skeletal development (Bicknell et al, 2005; Krakow et al, 2004), the molecular mechanism by which Filamins influences bone development remains unknown.

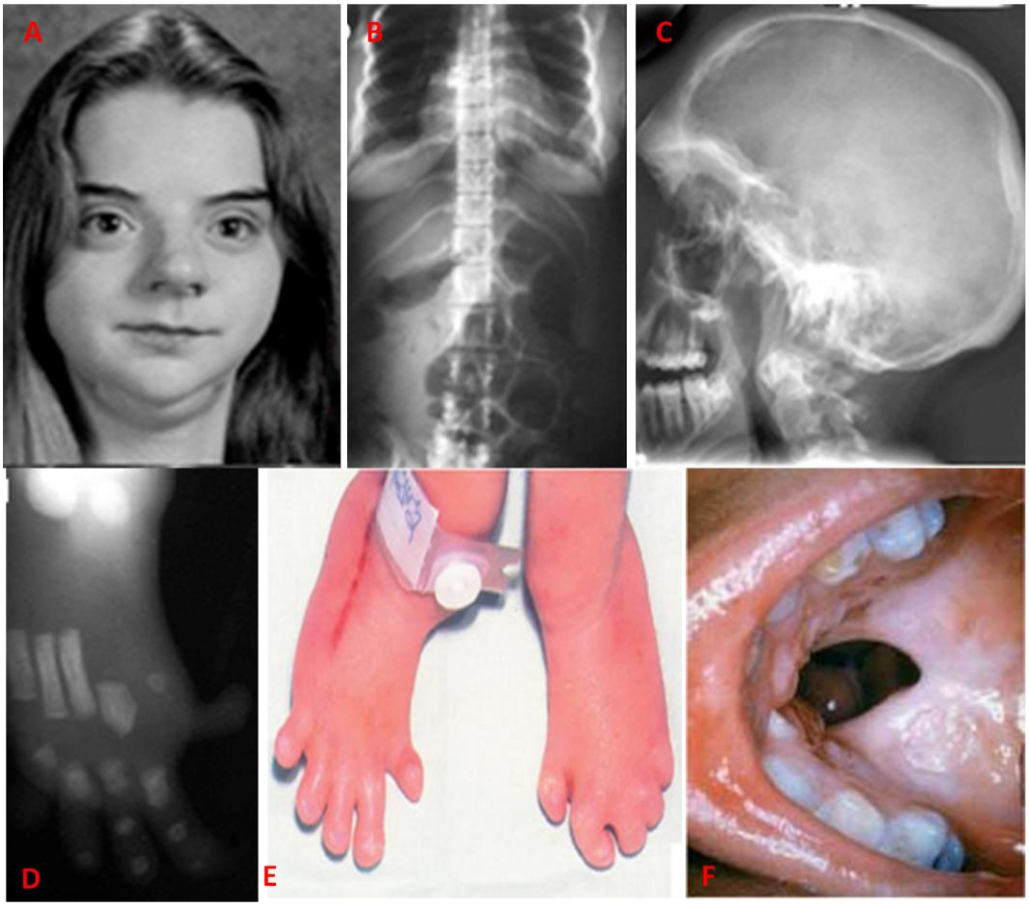


Fig. 1.9 The clinical manifestations caused by mutations in Filamin A. Melnick Needles Syndrome (a,b), Frontometaphyseal Dysplasia (c), and Otopalotodigital Syndrome (d-f) are the most common cases in patients with Filamin A mutations. These diseases affect mainly the normal osteogenic process in humans. The most characteristic phenotype in those patients is the cranial malformation, failure of development in tarsal and metatharsal feet bones and cleft palate. Adapted from (Robertson, 2007)

1.15 Structure of Filamins

The three filamin isoforms in humans (filamin A, B and C) share 70% sequence identity (Feng & Walsh, 2004). Filamin A and Filamin B are ubiquitously expressed and Filamin C is found mainly in heart and skeletal muscles (Zhou, 2007). Both human and mouse Filamin A genes are located on the X chromosome, whereas human Filamin B and Filamin C are on autosomal chromosomes 3 and 7, respectively. All three filamin isoforms are widely expressed during development. In adults, Filamin A is the most abundant isoform and Filamin C expression is mainly restricted to skeletal and cardiac muscle cells (Stossel et al, 2001). The amino-terminal region of Filamins is composed of an actin-binding domain, which contains two calponin homology (CH) domains termed CH1 and CH2. Each of these CH domains is composed of 110 residues (Galkin, 2006) (Fig. 1.10). These residues were initially recognised in the protein calponin and they are present in a number of actin binding proteins, such as fimbrin, α-actin, utrophin, dystrophin and plectin as tandem pairs. The rest of the protein is made of semi-flexible 24 tandem repeats of ~96 amino acids each. These rod-like 24 repeats fold into anti-parallel ß-sheets, which resemble Ig-fold domains and function as interfaces for interactions with over 30 proteins of great functional diversity (Popowicz, 2006). There are two unique hinges of 35 residues, each positioned between repeats 15-16 and 23-24 which show great sequence divergence. The hinges add flexibility to the filamin structure and are sites of proteolytic cleavage (Popowicz, 2006).

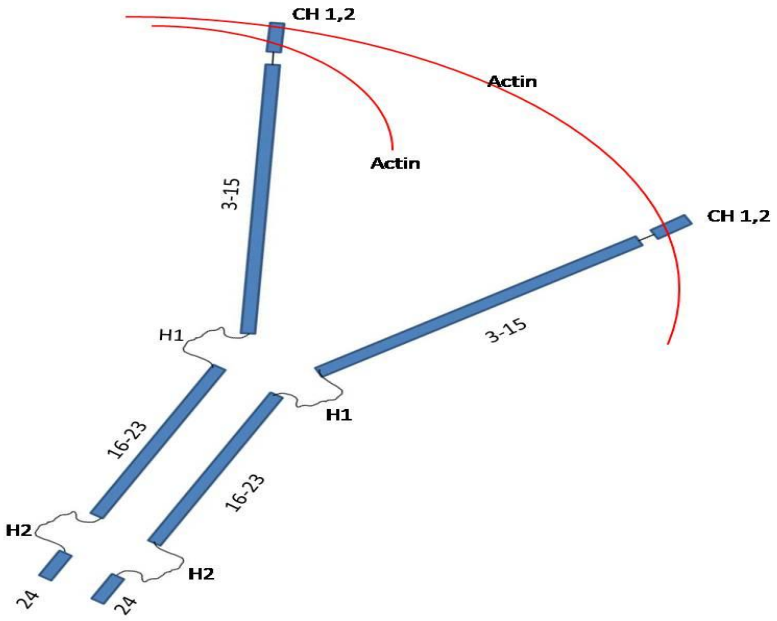


Fig. 1.10 The general structure of Filamins. The amino-terminal actin-binding domain of filamin contains two calponin homology domains depicted as CH1, 2. The rest of the protein is made of repeats that fold into anti-parallel ß-sheets. There is a stretch of 35 amino acids between repeats 15 and 16 as well as a second hinge between repeats 23 and 24. This figure represents Filamin A. The N-terminal actin-binding domain of Filamin A binds to filamentous actin (red) and efficiently tethers actin filaments into a three-dimensional gel. The last repeat is responsible for dimerisation which allows the formation of a V-shaped flexible structure. The anti-Filamin A antibodies used in this study recognise residues near the C terminal of human mouse and rat Filamin A (EP2405Y) and the full length Filamin A (CHEMICON).

1.15.1 Interaction of transcription factors with Filamin A

Filamin A interacts directly with several diverse proteins including receptors, kinases and transcription factors and has been proposed to act as a scaffold for signalling transduction pathways. Since 2000, reports have been published regarding the interaction between Filamin A and transcription factors (Popowicz, 2006). Those studies suggest that Filamin A/transcription factor interaction might regulate gene expression by, for example, modulating the subcellular localisation either in the nucleus and the cytoplasm of the specific transcription factor. Until now, the interactions between Filamin A and molecules involved in transcription process have been confirmed: Androgen Receptor (Loy et al, 2003), Smads (Sasaki et al, 2001), CBFβ (Yoshida, 2005), FOXC1 (Berry et al, 2005) and P73α (Kim et al, 2007).

It has been shown that CBFβ, the cofactor on Runx2 associates with Filamin A and retains CBFβ in the cytoplasm which obstructs the association with the transcription factor Runx1. The interaction with Filamin A is mediated by the amino acids 68 to 93 of CBFβ and its deletion enabled CBFβ to translocate into the nucleus. Filamin A has been postulated to hold transcription factors in the cytoplasm from moving into the nucleus and therefore Filamin A plays a role in the regulation of signalling cascade (Yoshida, 2005).

Given the importance of Filamin A in osteal disease, the possible interaction between Filamin A and Runx2 and the functional mechanism by which this complex may regulate the osteoblastic process was explored.

1.16 Project Aims

Filamin A mutations have shown to produce bone malformation defects (Feng & Walsh, 2004). However, the mechanisms by which Filamin A impact on bone development are unknown. Runx2 is a transcription factor that has been shown to be a master regulator of bone formation and mutations of Runx2 in humans cause bone malformations (Ito, 2008). Both Filamin A and Runx2 knockout mice exhibit bone defects (Feng et al, 2006; Komori et al, 1997) and missense mutations of both genes have been found in patients who present skeletal abnormalities (Feng et al, 2006; Robertson et al, 2003). Previous studies in our group have shown that Filamin A can interact with both Runx2 and CBFβ by GST-pulldowns and immunoprecipitation of exogenous expressed proteins in HeLa cells. Therefore, the overall aim of this project was to determine the molecular function of the Filamin A/Runx2 interaction *in vivo*.

Initial experiments were designed to stablish whether the Filamin A/Runx2 complex exists in osteoblastic cells. Subsequently the role of Filamin A in Runx2-dependent gene regulation was investigated. Finally, expression microarray analysis was performed to determine potential target genes regulated by Filamin A.

2.0 Material and Methods

2.1 Cell culture

Cell lines were obtained from ATCC and were cultured in the stated media and incubated at 37°C with 5% CO2 (Table 2.0). For differentiation studies, MC3T3 cells were cultured in medium supplemented with 10 mM β-glycerol phosphate (Sigma) and 50 µg/ml L-ascorbic acid (Sigma). To block ribosomal DNA transcription SaOS-2 cells were incubated with 50 ng/ml of actinomycin D (AMD). Primary bone marrow cells were extracted from the tibial bone of healthy mice and were maintained in α-MEM medium containing 10% fetal bovine serum plus 2 mM L-glutamine and a penicillin-streptomycin cocktail.

2.2 Antibodies and plasmids.

Primary antibodies. Polyclonal anti-Runx2 (Calbiochem). The monoclonal or polyclonal anti-CBFβ antibody, polyclonal anti-Lamin B1, polyclonal (ab5127) or monoclonal (EP2405Y) anti-Filamin A, monoclonal anti-fibrillarin, monoclonal anti-nucleophosmin, monoclonal anti-RPA40, monoclonal anti-Nopp140, and monoclonal anti-UBF1, all were prurchased from AbCam. Monoclonal anti-Filamin A (Chemicon), monoclonal anti-β-tubulin (SIGMA), polyclonal anti-MMP-13, control rabbit and control mouse IgG (Millipore). **Secondary antibodes.** anti-rabbit or anti-mouse Alexa Fluor (Molecular Probes Invitrogen) and anti-rabbit or anti-mouse HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, UK).

Table 2.0 Culture conditions for mammalian cell lines

Cell Line	**Cell Line origin**	**Culture Media (GibcoBRL)** at 37°C with 5% CO_2
HeLa, HeLa S3	Human female, derived from a cervical carcinoma.	DMEM, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin.
M2	Human melanoma which does not express Filamin A.	Alpha MEM, 10% FBS 1% penicillin/streptomycin.
MC3T3	Mouse preosteoblast, derived from the calvaria. Vitamin-D_3 receptor positive.	Alpha MEM, 10% FBS, 2mM L-glutamine, 1mM sodium pyruvate, 1% penicillin/streptomycin.
A7	Human melanoma cell line. A clonal derivate from M2 cells which stably express Filamin A.	Alpha MEM, 10% FBS 1% penicillin/streptomycin.
SaOS-2 andU2-OS	Human osteoblastic cell lines derived from an osteosarcoma.	McCoy's medium, 15% FBS, 2 mM L-glutamine 1% penicillin-streptomycin.
LNCaP	Prostate cancer cell line, extracted from a lymph node.	RPMI 1640, 10% FBS 1% penicillin/streptomycin.
C2C12	Mouse Myoblatic cell line	DMEM, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin.
PC3	Prostate cancer cell line, isolated from a bone metastasis.	DMEM, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin.

Construct plasmids. a) The pGL3MMP-13 promoter reporter plasmid, encoding the wild-type human promoter sequence, has been described previously (Mengshol et al, 2001). pGL3MMP-13mut was generated by mutating the distal Runx site in the pGL3MMP-13 promoter already harbouring a mutation in the proximal Runx-binding site (Mengshol et al, 2001), using the QuickChangeXL Site directed Mutagenesis Kit (Stratagene, CA). The subsequent plasmid has both proximal and distal Runx-binding sites mutated. The sequence was verified by di-deoxy sequencing. **b)** The ribosomal DNA promoter reporter plasmid (rDNAp), encoding the wild-type mouse promoter sequence, has been described previously (Budde & Grummt, 1999). rDNAp contains a proximal Runx site in its sequence. rDNApMut was generated by mutating the Runx binding-site in the rDNAp from TGAGGT to TGAACT using the QuickChangeXL Site directed Mutagenesis Kit (Stratagene, CA). The sequence was verified by di-deoxy sequencing. **c)** pcDNA3-Filamin A-myc and PCDNA3-Filamin A-GFP plasmids were provided by Dr John Blenis and Dr David A. Calderwood, respectively. The sequence of these plasmids was verified by di-deoxy sequencing. **d)** The construct plamid encoding the Runx2 sequence (pCMV5-Runx2) was obtained from Karenty's group (Ducy et al, 1997) and the sequence was verified by di-deoxy sequencing.

2.3 siRNA and Plasmid DNA transfection

Cells at 60% confluency were transfected using Oligofectamine transfection reagent (Invitrogen) with a pool of small interfering RNA (siRNA) specific for human or mouse Filamin A (Santa Cruz, CA). Non-specific siRNAs (Santa Cruz, CA) were transfected in control cells. Opti-MEM (Invitrogen) was used to dilute the siRNA duplexes and Oligofectamine for transfection. After treating the cells with siRNA for 4 h, the cells

were supplemented with growth media with FBS (final concentration of 15%). The siRNA experiment was carried out for 4 or 5 days, at which time the cells were harvested for total protein and RNA isolation to analyze the knockdown effect of Filamin siRNA and used for experiments.

Cells at 80% confluency were transfected with 1 ug of the DNA plasmid constructs using LipofectAMINE 2000 transfection reagent (Invitrogen), according to the manufacturer's instructions. Control cells were transfected in parallel with the same amount of empty vector DNA. Most of the transfection were perfomed in 6 well plates exept for microarray experimients in which 10 mm plates were used. All transfections were performed in triplicate. RNA and protein were harvested at 24 to 48 h to either assess DNA promoter-driven reporter activity, or Runx2 and Filamin A protein levels.

2.4 Site directed mutagenesis

Mutations in the MMP-13 promoter plasmid were specifically introduced using using the QuickChangeXL Site directed Mutagenesis Kit following the manufacturer's protocol (Stratagene). The site specific mutations were introduced into the plasmid using mutated primers, which were present in the following PCR reaction; 5ng of template DNA, 125 ng of each primer, 20 nmol of dNTPs, 5 μl 10×Pfu reaction buffer, 1 μl pfu turbo (2.5U/μl) and the volume of each reaction was adjusted to a total of 50μl using dH2O. Each PCR underwent an initial DNA denaturing step at 95°C for 30 s, then 12 cycles at the following temperatures, 95°C for 30 s, 55°C for 1 min, and 68°C for 12 min. After completion of the 12 thermo-cycles, 1μl of DpnI was added to each reaction and then incubated at 37°C for 1 hr. XL-1 blue Epicurian coli super competent

cells (Stratagene) were then transformed with 1µl of the DpnI treated PCR mixture in accordance with the manufacturer's protocol.

2.5 Transformation of bacteria

Bacteria were made competent by growing a 5ml culture of bacteria until it reached an optical density of 0.6 when measured at a wavelength of 600 nm. The bacteria were then centrifuged at 10,000 rpm for 1 min. The pellet was resuspended in 300 µl of TFBI [10 mM MOPS; 10 mM Rubidium Chloride; pH 7.0] and incubated at 4˚C for 10 mins. The bacteria were then centrifuged at 10,000 rpm in a micro centrifuge for 1 min and the pellet resuspended in 200µl of TFBIII [100 mM MOPS; 50 mM $CaCl_2$; 10 mM KCl; pH 6.5]. To transform the bacteria, approximately 0.1µg of plasmid DNA was added to the bacteria and the cells were heat shocked for 2.5 min at 42˚C. The bacteria were then plated on selected LB plated and incubated overnight at 37˚C.

2.6 Assesment of reporter activity

Cells at 60% confluency were transfected in 6 well plates. For transfections performed in 6 well dishes each transfection contained 900 ng of the indicated reporter plasmid and 100ng of Renilla luciferase reporter, pRL-SV40, which was used to normalise for transfection efficiency. 5 h post-transfection the transfection mixture was removed and replaced with the standard culture media for that cell line. Either 24 or 48 h post transfection the cells were lysed and the luciferase activity was determined using the dual luciferase reporter assay system according to the manufacturer's recommendations (Promega). All transfections were performed in triplicate and data is presented as mean

values with standard deviation. Values are relative to the activity of either pGL3-Basic or pGL3-Promoter.

To asses the reporter activity of rDNAp and rDNApmut, RNA and protein were harvested at 24 h. Primers were designed against the pUC9 transcribed sequence which is contained in the rDNAp. Reporter activity was determined by RT-qPCR and was normalized to GAPDH gene.

2.7 Total Cell extracts

Total cell extracts from mammalian cells were routinely prepared from 5×10^6 cells. 1 ml of 1X Loading Buffer [0.625M Tris-Cl (pH 6.8), 50% glycerol, 10% SDS, 10% ß-mercaptoethanol, 0.05% bromophenol blue] was added to each plate, cells were scraped and transferred to a 1.5 ml tube. Following 5 min boiling at 100°C, 10 µl of each sample was loaded into a SDS-Polyacrylamide Gel Electrophoresis (PAGE). The rest of the samples were aliquoted and stored at -80 °C.

2.8 Isolation of nuclear and cytoplasmic protein fractions

Nuclear extracts from mammalian cells were routinely prepared according to the method described in Schreiber et al. 1989. 5×10^6 cells were pelleted by centrifugation at 1,000 rpm for 5 min. The cells were washed with 3 ml of 1X PBS [137mM NaCl; 2.7mM KCl; 1.5mM Na_2HPO_4; 1.8mM KH_2PO_4] and re-pelleted. The pellet was resuspended in 1ml 1X PBS and transferred to an eppendorf tube where it was pelleted in a micro centrifuge for 1 min at 13,000 rpm. The supernatant was removed and the pellet was resuspended by gentle pipetting in 400 µl of ice cold Buffer A [10mM HEPES pH7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM

PMSF]. To this 40 μl of complete mini EDTA-free protease inhibitor cocktail (Roche) was added and the cells were incubated at 4°C for 15 min to allow the cells to swell. 25 μl of 10% Nonidet NP-40 (Fluka) was added and the cells vigorously vortexed for 10 s. The lysed cells were then centrifuged at 4°C, 14,000 rpm for 30 s. The supernatant containing the cytoplasm and RNA was saved in another tube and the nuclear pellet was resuspended in 50 μl of ice cold buffer B [20mM HEPES pH 7.9; 0.4M NaCl; 1mM EDTA; 1mM EGTA; 1mM DTT; 1mM PMSF]. 5 μl of protease inhibitor cocktail was added and the tube was vigorously vortexed for 60 min at 4°C. Nuclear debris was pelleted by centrifugation at 14,000 rpm at 4°C for 5 min, and the supernatant containing the nuclear proteins was collected and immediately used for the experiments. Alternatively, for immunofluorescence or western blot analysis, nuclei were purified using the Nuclei EZ Prep Nuclei Isolation Kit (SIGMA). Cells were passed several times in a Dounce homogenizer and nuclei were centrifuged through a dense sucrose cushion (Greenberg and Bender, 2007). Nuclear and cytoplasmic fractions were subjected to SDS-PAGE and western blot.

2.9 Purification of Nucleoli

Cells were harvest cells by trpysinisation and washed 3X with ice-cold PBS at 218 g (1000 rpm, Beckman GS-6 centrifuge, GH-3.8 rotor). The protocol was always maintained at 4°C. After the final PBS wash, cells were resuspended in 5ml of Buffer A (10 mM Hepes, pH 7.9, 10mM KCl, 1.5mM $MgCl_2$, 0.5mM DTT) and incubated on ice for 5 min. Cells were transferred to a pre-cooled 7 ml Dounce tissue homogenizer (Wheaton Scientific Product Cat no: 357542) and homogenized 10 times using a tight pestle. Cells were centrifuged at 218g (1000 rpm for 5 min. and resuspended with 3 ml S1 solution (0.25 M Sucrose, 10 mM $MgCl_2$). The resuspended pellet was layered over 3 ml of S2 solution and centrifuged at 1430 g for 5 min. The pellet was resuspended in 3m of S2 solution (0.35 M Sucrose, 0.5 mM MgCl2) and sonicated for 6 bursts (with 10 second intervals between each burst) using a Misonix sonicator fitted with a micro tip probe and set at power setting 5. The sonicated sample was layered over 3 ml of S3 solution (.88 M Sucrose, 0.5 mM MgCl2) and centrifuged at 3000g (12,000 rpm) for 10 min. The pellet containing the isolated nucleol was washed with solution S2 and centrifuged at 1400 rpm twice. Until this stage the pellet contained highly purified nucleoli.

2.10 Western Blotting

Samples were denatured by mixing protein sample 4:1 with a 5X concentration of loading buffer [0.625M Tris-Cl (pH 6.8), 50% glycerol, 10% SDS, 10% ß-mercaptoethanol, 0.05% bromophenol blue]. The samples were heated at 100°C for 5 min prior to loading onto the gel. 250 volts was used for running gels and the time was adjusted according to the percentage of polyacrylamide gel used and the protein molecular weights of interest. A molecular weight marker was loaded in parallel. The gel and the nitrocellulose membrane were equilibrated in transfer buffer [250mM Tris, 190mM Glycine, Methanol] for 15 min, and semidry transfer was performed by using a BlotterTM (Bio-Rad) at 12 Volts for 1.5 h on 280 kDa proteins, and 70 min on 66 kDa and below. The blot was then blocked with 5% non-fat dried milk (Marvel) freshly made in Tris Buffer Saline (TBS) containing 0.05% TWEEN-20 (Sigma) for 2 h. The membrane was then probed with the indicated primary antibody for 2 hrs at room temperature or 4°C overnight on a rotating shaker. All primary antibodies were probed at 1:1000 dilution, exept for the mouse monoclonal anti CBFβ antibody (1/500 dilution). The blot was then rinsed 5 times in TBS-0.05% TWEEN 5 minutes each at room temperature. Goat anti-mouse IgG or goat anti-rabbit IgG, which are conjugated with horseradish peroxidase (Pierce), were used to probe the blot at a 1/5000 dilution in TBST/5% non-fat milk for 30-60 min. The membrane was again rinsed 5 times with TBS-0.05% TWEEN to wash off excess of secondary antibody for 5 min each. Immune complex were then detected by using the Supersignal West Dura Extended Duration Substrate (Pierce), following manufacturer recommendations. Bio-Rad Fluor-S multi-imager with Quantity One software was used to detect the signal

2.11 Immunoprecipitation

Total cell extract preparations were pre-clear with either Protein A or B agarose beads, washed and then incubated overnight at 4°C with 2-5 µg of specific antibodies followed by 2 h incubation with Protein G or Protein A agarose beads. Normal mouse or rabbit IgG was used as a control. After this incubation, the beads were washed 5 X, 10 min each at 4°C with lysis buffer [1% Triton, 20 mM Tris-HCl, pH 7.5, 150mM Sodium Chloride, glycerol 10% (v/v)] and the last wash with RIPA buffer [25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS]. Washed beads were boiled with 36 µl of Loading Buffer [0.625M Tris-Cl (pH 6.8), 50% glycerol, 10% SDS, 10% ß-mercaptoethanol, 0.05% bromophenol blue]. 12 µl of the immunoprecipitate was loaded to an 8-10% SDS-PAGE and Western blot was performed.

2.12 Immunofluorescence analysis

Cells grown on cover slips were fixed with 4% paraformaldehyde, permeabilised with 0.1% Triton X-100, and blocked with 1% bovine serum albumin in phosphate-buffered saline (PBS). Then, cells were incubated with the primary antibody (1:1000 dilution for all the antibodies) in PBS-0.5% TWEEN 20 for 1 hr at room temperature. After the incubation, cells were washes 3X with PBS-0.5% TWEEN 20 for 5 min each, followed by the secondary antibody of Alexa fluor at a dilution of 1:250. Cells were washed 3X with PBS and nuclear staining was perform along with mounting with Prolong® Gold antifade reagent with DAPI (4,6-diamidino-2-phenylindole) (Molecular Probes, Invitrogen) following manufacture's protocol. The cover slips were allowed air-dry overnight at room temperature before examined under microscopy examination.

Depending of the nature of the experiment a snapshot fluorescence microscope, or a confocal microscope was used. For normal fluorescence microscopy, Images were collected on a Olympus BX51 upright microscope using a 40-100X/ 0.30 Plan Fln objective and captured using a Coolsnap ES camera (Photometrics) through MetaVue Software (Molecular Devices. Images were then processed and analysed using ImageJ (http://rsb.info.nih.gov/ij). Images were processed using MacBiophotonics ImageJ and Imaris software. For confocal analysis, single optical sections were captured through a confocal microscope (Zeiss LSM 510) equipped with a plan apochromat 60-100X objective (DIC 1.4NA). Images were processed using Zeiss and Imaris (Bitplane) software. For deconvolution analysis, a Deltavision microscope was used with a 100x objective was used. Images were deconvolved using 5–10 iterations and pre-filter cut-off values (microns) of 0.05. Images were processed using Zeiss and Imaris (Bitplane) software.

2.13 Polymerase Chain Reaction (PCR).

Total RNA was prepared from cells using the RNeasy Mini Kit (Qiagen) under manufacturer protocol and recommendations. 100 ng of each sample was used in triplicate in a quantitative reverse transcription-PCR (RT-qPCR) using a one step Quantitect SYBR green RT-PCR kit (Qiagen). Primers were designed to amplify approximately 100 bp target fragments for each gene in study. The device used to perform RT-qPCR was the CFX-96 termocycler (BIORAD). The PCR conditions were the same as stated in the Quantitect SYBR green RT-PCR protocol (Qiagen). To assess the relative changes in gene expression for each target gene in Filamin A-siRNA transfected cells to that of the control-siRNA group the $2^{-\Delta\Delta ct}$ method was used. The 2-

$^{\Delta\Delta ct}$ method is a convenient way to analyze the relative changes in gene expression from RT-qPCR experiments based in the comparison between the Ct values of one target gene to a reference gene. Changes in mRNA expression levels were relative to GAPDH mRNA.

Table 2.1 Primers used for RT-qPCR experiments.

Accession #	HUMAN Gene	Abr.	Primer Forward	Primer Reverse
NM_000478	alkaline phosphatase, liver/bone/kidney	ALP	ATG GGA TGG GTG TCT CCA CA (20 bp)	CCA CGA AGG GGA ACT TGT C (19 bp)
NM_001718	bone morphogenetic protein 6	BMP-6	TTT TGT TGG ACA CCC GTG TAG (21 bp)	CTG AAG CCC CAT GTT ATG CTG (21 bp)
NM_004995	matrix metallopeptidase 14	MMP-14	CGA GGT GCC CTA TGC CTA C (19 bp)	CTC GGC AGA GTC AAA GTG G (19 bp)
NM_012383	osteoclast stimulating factor 1	OSF	CTG GTT GAG AGA GTG TTT GGA C (22	CTT CCA GCT TTG TCT AAG CCA T (22 bp)
NM_004530	matrix metallopeptidase 2	MMP-2	CTT CCA AGT CTG GAG CGA TGT (21 bp)	TAC CGT CAA AGG GGT ATC CAT (21 bp)
NM_005450	Noggin	NOG	GAA GCA GCG CCT AAG CAA GA (20 bp)	CTG CCC ACC TTC ACG TAG C (19 bp)
NM_001202	bone morphogenetic protein 4	BMP-4	TGG TCT TGA GTA TCC TGA GCG (21 bp)	GCT GAG GTT AAA GAG GAA ACG A (22 bp)
NM_000393	collagen, type V, alpha 2	Col5a2	AAA TAG CCT GCA CTC AGA ATG G (22	CAG ATC ACG TCA TCG CAC AAC (21 bp)
NM_005222	distal-less homeobox 6	Distbox6	CCT TAC CTC CAG TCC TAC CAC (21 bp	AGG CTT CCG AAT CTT TTT CCC (21 bp)
NM_001024847	transforming growth factor, beta receptor II	TGB-beta	TTC AGA AGT CGG ATG TGG AAA TG (2	GTT GTC AGT GAC TAT CAT GTC GT (23 bp)
NM_152860	Sp7 transcription factor, Osterix	OSX	CCT CTG CGG GAC TCA ACA AC (20 bp)	TAA AGG GGG CTG GAT AAG CAT (21 bp)
NM_001200	bone morphogenetic protein 2	BMP-2	ACT ACC AGA AAC GAG TGG GAA (21 bp	ATC TGT TCT CGG AAA ACC TGA AG (23 bp)
NM_021073	bone morphogenetic protein 5	BMP-5	AAG GGT ATT GTG GGT TTC CTC T (22 b	TCG TGG TTC CGT AGT CTT CTA T (22 bp)
NM_005221	distal-less homeobox 5	Dbox5	GAG TGT TTG ACA GAA GGG TCC (21 bp	GAA TCG GTA GCT GAA GAC TCG (21 bp)
NM_001005340	glycoprotein (transmembrane) nmb	GNMB	TTT CCA GAA ATT GGG ACG ATG TT (23	GTC ACT TCC ATG AGT TGA GGC (21 bp)
NM_002449	msh homeobox 2	Msx2	CAC CCT GAG GAA ACA CAA GAC (21 bp	AAC TCT GCA CGC TCT GCA AT (20 bp)
NM_198965	parathyroid hormone-like hormone	PTHorm	ATT TAC GGC GAC GAT TCT TCC (21 bp)	CGG ACG GGG TGG ATT TCA G (19 bp)
NM_001011658	trafficking protein particle complex 2;	TPPC2	GAC CAT CGT CAT CTG AAC CAG (21 bp	CCG ACA CAA ACC ACT CGT T (19 bp)
NM_020127	tuftelin 1	Tuftelin	GCG GGC AGG AAG ACC TAT G (19 bp)	GGG ACT CCA CCA GTT CTG AAG (21 bp)
NM_001901	Conective Tissue Growth Factor	CTGF	TTG GCC CAG ACC CAA CTA TG (20 bp)	CAG GAG GCG TTG TCA TTG GT (20 bp)
NM_001110556	Filamin Alpha	Fln A	CTG TGG CTT CTC CGT CTG G (19 bp)	TGT GAC ATA GCA CTC CTC CAG G (22 bp)
Accession #	MOUSE Gene	Abr.	Primer Forward	Primer Reverse
NM_001161456	Core Binding Factor beta	CBFβ	GTA TGG GTT GCC TGG AGT TTG A (22 b	GTC TTC TTG CCT CCA TTT CCT C (22 bp)
NM_001145920	Runt related transcription factor 2	Runx2	AAA TGC CTC CGC TGT TAT GAA (21 bp)	GCT CCG GCC CAC AAA TCT (18 bp)
NM_001037939	Osteocalcin	OCN	CCG GGA GCA GTG TGA GCT TA (20 bp)	GTA GGC GGT CTT CAA GCC ATA CT (23 bp)
NM_001204201	Osteopontin	OSP	GAT GCC ACA GAT GAG GAC CT (20 bp)	CAG AGG GCA TGC TCA GAA GC (20 bp)
NM_009242	Osteonectin	ON	AAT CCA GAG CTG TGG CAC ACA (21 bp	TGG AAA GAA ACG CCC TAA GAG TCG (24 bp)
NM_008607	Matrix Metalloproteinase-13	MMP-13	ATG ATG ACG TTC AAG GAA TTC AG (2	CTG GTA ATG GCA TCA AGG GAT AG (23 bp)
NM_010227	Filamin Alpha	Fln A	ATA TCC GTC TCA GTC CTT (18 bp)	CAC CAG TCT TCT CCA ATC (18 bp)
NM_002046	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	GCA CAG TCA AGG CCG AGA A (19 bp)	GCC TTC TCC ATG GTG GTG A (19 bp)

2.14 Chromatin immunoprecipitation assays (ChIP)

ChIP assays were performed as described previously (Deng & Roberts, 2007). RT-qPCR was performed with promoter primers for MMP-13, OPN, rDNA genes. GAPDH primers were used as the reference. The relative enrichment of target DNA was determined by calculating the ratio of DNA from specific antibody to that from control IgG and normalized by GAPDH non-specific genomic DNA within the same sample.

2.15 cDNA Microarray Analysis

Micoarray analysis was performed in triplicate, M2 and A7 cells were grown until 80% confluency in 100mm plates and SaOS-2 cells were transfected with either a Filamin A specific siRNA or a scrambled siRNA as a control, they were grown until 80% confluency in 100 mm plates. After harvesting the cells, the cell lysates were passed through a RNeasy column in order to purify the RNA. The concentration of mRNA was measured and quality and control of the RNA was assesed. Right after cDNA was prepared, the microarray experiments were performed in the microarray facility by using the Affymetrix GeneChip® Human genome U133 array Plus 2.0 array.

Once the experiment was made, the quality of the data was assessed and then processed to calculate the differential expression by statistical analysis. The final step was a list of genes that were arranged depending on the fold change versus the control samples. A negative fold change represented the downregulated genes and the positive fold change represented the upregulated genes. The genes that felt into -1.25 to 1.25 fold change were considered as the unaffected genes.

3.0 Filamin A is nuclear and interacts with Runx2 in bone cells

3.1 Introduction

Filamin A is a 280 kDa protein that cross-links actin into networks and stress fibres. It consists of an amino-terminal actin-binding domain and 24 repeats of Ig-like domains linked to form a rod-like structure that dimerises to cross link actin (Stossel et al, 2001). Filamin A is widely expressed, interacts directly with several diverse proteins and has a role as a molecular scaffold (Feng & Walsh, 2004).

The transcription factor Runx2 is a master-regulator of bone development (Stein et al, 2004). Runx2-null mice fail to develop a skeleton due to defects in osteoblast and chondrocyte differentiation (Komori et al, 1997; Otto et al, 1997). Mutations in Runx2 also cause the skeletal disorder cleidocranial dysplasia. Filamin A is known to retain the Runx-coactivator, CBFβ, in the cytoplasm (Yoshida, 2005). However, the role of Filamin A in regulating Runx2 and its target genes has not been addressed.

Here, it is shown that Filamin A interacts directly with Runx2 and that Filamin A is abundantly expressed in the nucleus of osteoblastic cells. Also, it is demonstrated that Filamin A is recruited to the promoter of the gene encoding the bone matrix-degrading enzyme, matrix metalloproteinase-13 (MMP-13), and suppresses its Runx2-dependent expression. Moreover, during differentiation of osteoblastic cells Filamin A is downregulated and MMP-13 is upregulated.

3.2 Filamin A interacts directly with Runx2

In order to determine whether endogenous Filamin A and Runx2 interact it was first established that they are both expressed in osteoblastic cells. Western blot analysis was performed on total cell extracts from the mouse osteoblastic cell line MC3T3 and in the human osteosarcoma cell line, SaOS-2. These cell lines are widely used to study osteoblast differentiation and gene expression (Fig. 3A) and they have previously been shown to express Runx2 (Young et al, 2007). HeLa cells were used as a negative control for Runx2 expression. Runx2 expression was confirmed by incubating immunoblots containing total cell extracts with an anti-Runx2 antibody. As expected a band of approximately 64 kDa was observed in the osteoblast cell lines but not in HeLa cells (Fig. 3B, left panel). Incubation with an anti-Filamin A antibody revealed a band of approximately 280 kDa, thus confirming expression of Filamin A in the osteoblast cells (Fig. 3 B, right panel). However, it seems that mouse and human Filamin A display different cleavage products. The additional, lower molecular weight bands are most likely to be proteolytic cleavage products derived from the full-length protein. After confirming endogenous expression of the proteins in the osteoblast cell lines co-immunoprecipitation experiments were performed to determine if the endogenous Filamin A and Runx2 proteins interact *in vivo*. Co-immunoprecipitation experiments were performed using cell lysates from SaOS-2 and MC3T3 cells. The interaction between Runx2 and the cofactor CBFβ has been widely established in osteoblastic cell lines (Komori, 2003) and in the metastatic breast cancer cell line MDA-MB-231 (Mendoza-Villanueva et al, 2010). The interaction between Filamin A and CBFβ has been shown in melanoma cell lines but not in osteoblastic cells (Yoshida, 2005). As

expected, the Runx2-immunoprecipitated fraction subjected to western blot for CBFβ shows that CBFβ is associated with Runx2 (Fig. 3.1A left panel, lane 2). Furthermore, immunoprecipitation of Filamin A and subsequent western blotting confirmed that CBFβ is associated with Filamin A (Fig. 3.1A, right panel, lane 4). Having confirmed previously reported protein interactions the next experiment was designed to examine the putative interaction between Runx2 and Filamin A.

Filamin A was immunoprecipitated from MC3T3 and SaOS-2 cell lysates and subjected to both Filamin A and Runx2 detection by western blot. Filamin A was present in the Filamin A-immunoprecipitated fraction thus indicating the suitability of Filamin A antibody for immunoprecipitation experiments (Figure 3.1B, upper panels, lane 2 and 5). Immunoprecipitation of Filamin A and subsequent western blotting demonstrated that Runx2 is associated with Filamin A in osteoblast cells (Figure 3.1B, lower panels, lane 2 and 5). This is the first demonstration that endogenous Filamin A is physically associated with endogenous Runx2 *in vivo*.

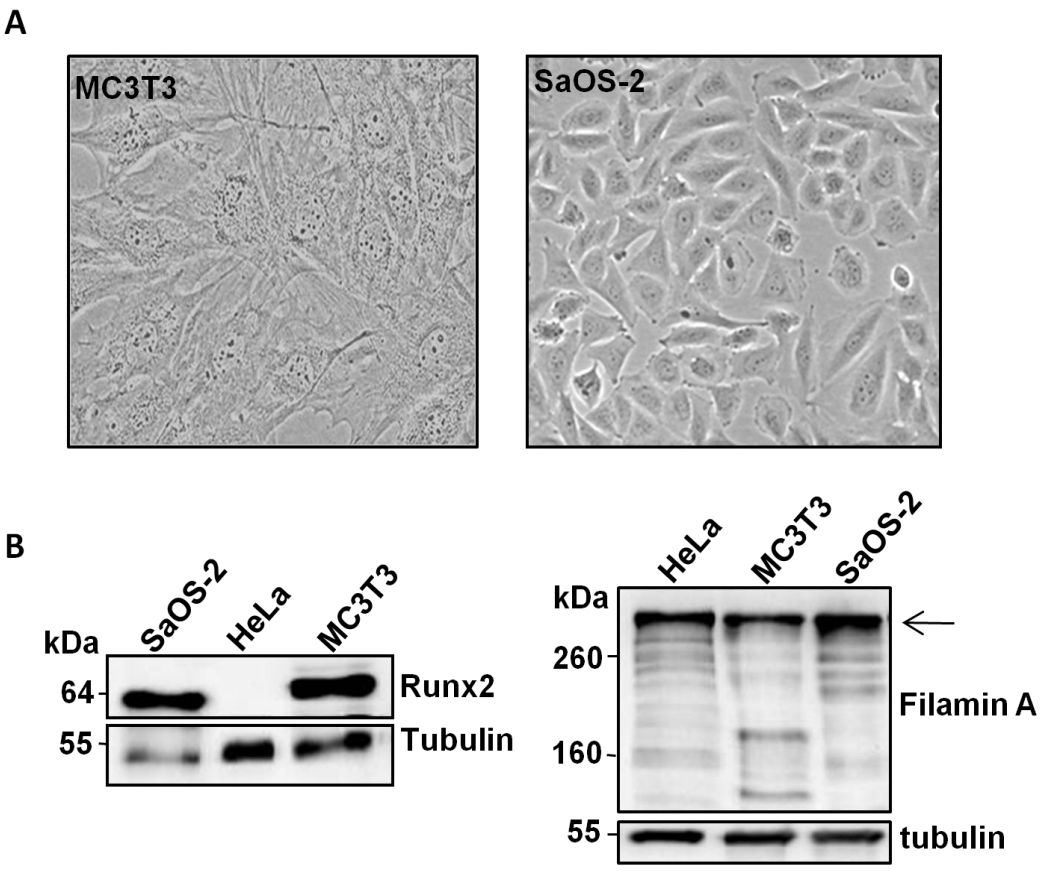


Fig. 3.0 Endogenous expression of Runx2 and Filamin A in mouse and human osteoblastic cell lines. (A) Light microscopy shows morphological characteristics in mouse MC3T3 and human SaOS-2 cells. (B) Western blot analysis of total cell extracts against Runx2 and Filamin A (left and right panel, respectively). Molecular mass is indicated on the left, the cell type is indicated above the lanes, and the identity of the proteins is indicated on the right. Full length Filamin A is indicated with an arrow. Tubulin was used as a loading control.

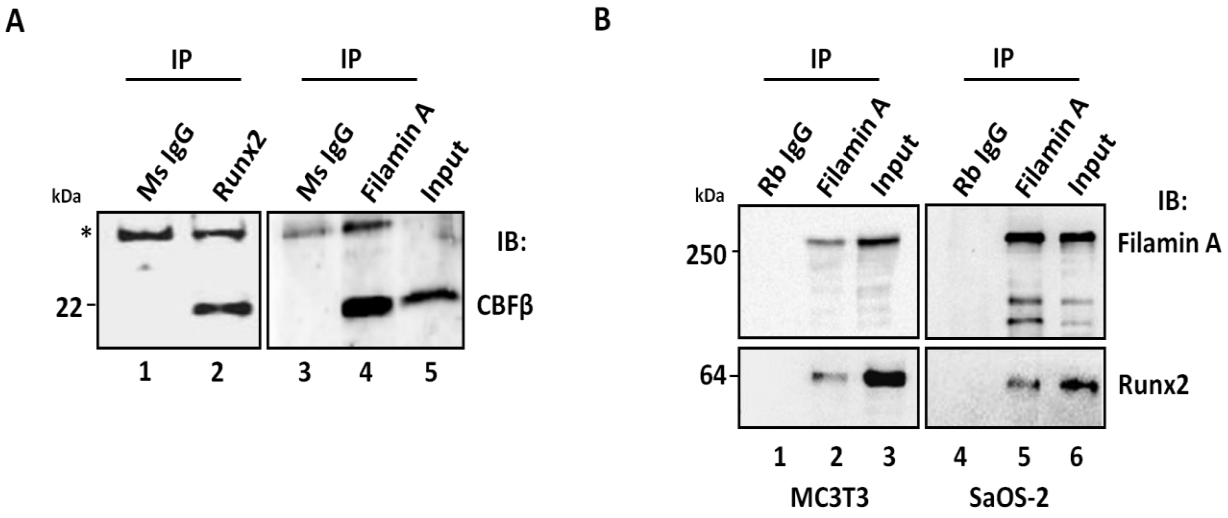


Fig. 3.1 Filamin A interacts with Runx2. (A) Co-immunoprecipitation (co-IP) showing that CBFβ associates with Runx2 and Filamin A in MC3T3 cells. Immmunoprecitation (IP) was performed on MC3T3 cell lysates using antibodies as indicated above the lanes, followed by immunoblotting with anti-CBFβ antibody. Mouse IgG (MsIgG) was used as a control. IgG chain is denoted by *. (B) Runx2 is associated with Filamin A in osteoblastic cells. Filamin A was immunoprecipitated with an anti-Filamin A antibody followed by immunoblotting with an anti-Filamin A antibody (upper panels) or an anti-Runx2 antibody (lower panels). Rabbit IgG (RbIgG) was used as a control in IP experiments.

3.3 Filamin A and Runx2 co-localise to the nucleus

Having demonstrated that Runx2 interacts with Filamin A, immunofluorescence microscopy experiments were designed to determine where Filamin A and Runx2 interact within the cell. When stained with an anti-Runx2 antibody staining was almost exclusively nuclear in the cells tested (Fig. 3.2 A, green). A lower level of staining was also observed in the cytoplasm. Staining with the Filamin A antibody "ab5127" revealed that Filamin A is not only present in the cytoplasm, where it associates with cytoskeletal fibres, but it is also expressed in the nucleus (Fig. 3.2 A and Fig. 3.2 B, red panels, when compared to β-tubulin staining (Fig. 3.2 B, green) which is exclusively cytoplasmic). Merging the stained images demonstrated that Filamin A and Runx2 co-localise to the nucleus (Fig. 3.2 A, overlay panels). In order to confirm the previous observation, western blot analysis of nuclear and cytoplasmic extracts were performed in MC3T3 and SaOS-2. Western blot analysis shown that Filamin A, as well as Runx2, is expressed in the nucleus of both cell lines and that the predominant species of Filamin A is 280 KDa, which corresponds to the full-length Filamin A (Fig. 3.2C). Moreover, we extended immunoblot analisis to different cell-types including osteoblastic (SaOS-2 and U2-OS), prostate cancer (PC3 and LNCap), cervical cancer (HeLa and HeLa-S3) and myoblast (C2C12) cell lines (Fig. 3.2 D). All the nuclear cell extracts, but LNCaP cells, showed full length Filamin A expression (Fig. 3.2 D, lane 6). The purity of the cell extracts in C and D was confirmed by immunoblotting for Lamin B1, which is part of the nuclear envelope and β-tubulin, which is found exclusively in the cytoplasm. It is therefore likely that the interaction between Runx2 and Filamin A occurs in the nucleus.

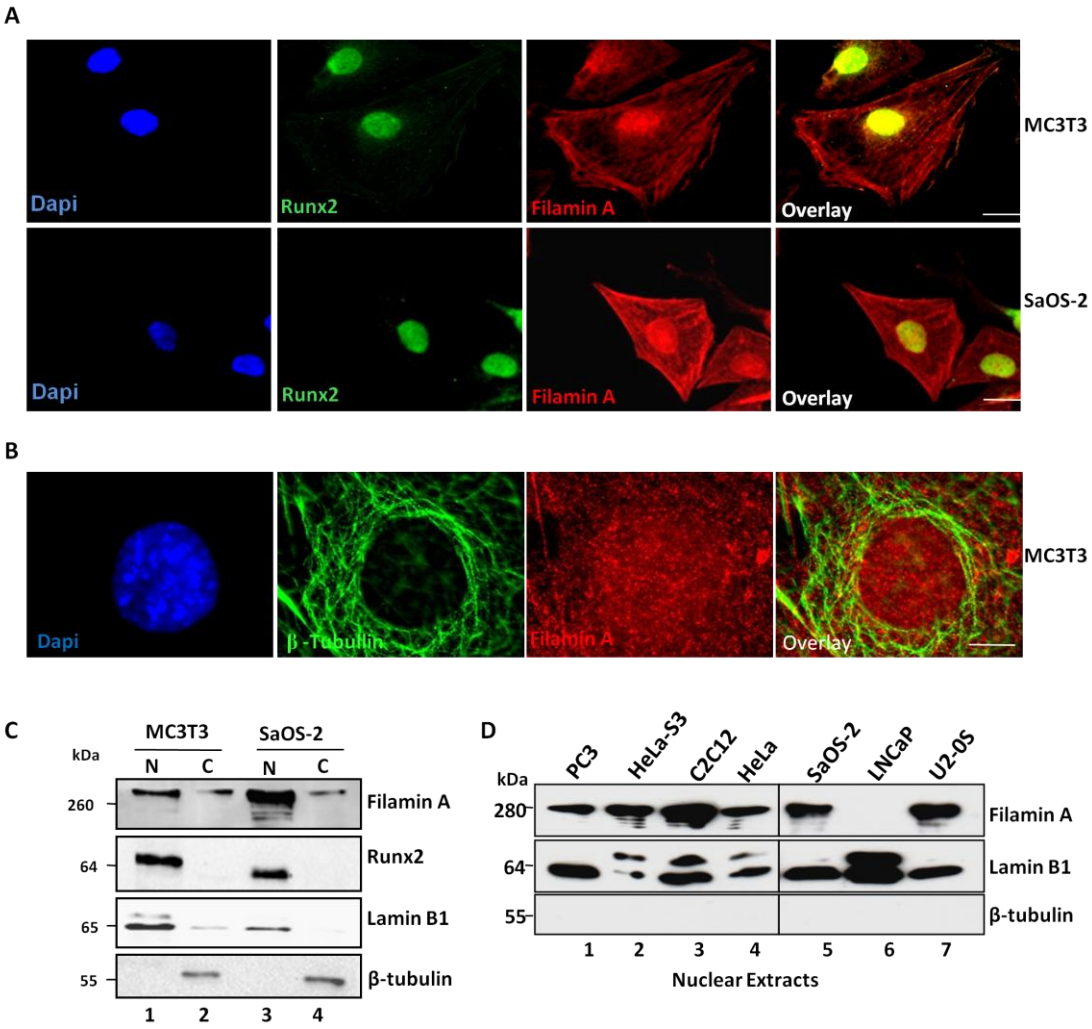


Fig. 3.2 Filamin A and Runx2 co-localise to the nucleus. (A) Confocal microscopy showing Runx2 and Filamin A in the nucleus. MC3T3 and SaOS-2 were immunostained as indicated in the panels. Nuclei (blue) were stained with DAPI. Bar, 10μM. (B) Confocal microscopy in MC3T3 cells showing the typical cytoskeletal β-tubulin fibres which stains only the cytoplasmic surface, in comparison with the Filamin A staining which stained cytoskeleton fibres as well as the nucleus. Bar, 5 μM. (C) Western blot showing Filamin A and Runx2 in nuclear extracts. Nuclear (N) and cytoplasmic (C) extracts immunoblotted with antibodies as indicated. (D) Western blot in nuclear extracts from different cell-types showing nuclear localisation of Filamin A, exept in LNCaP cells. Molecular mass is indicated on the left.

3.4 Establishing the knockdown of Filamin A

In order to determine the role of the Filamin A/Runx2 complex experiments were performed to determine the effect of knocking down Filamin A on the localisation of Runx2 and on the expression of Runx2-target genes. Initial experiments established a protocol to knockdown Filamin A expression by transfecting siRNA targeting Filamin A in the osteoblastic MC3T3 and SaOS-2 cell lines. Transfection of MC3T3 cells with Filamin A-siRNA reduced Filamin A expression from day 1, and maximum knockdown of approximately 80% was achieved 4 days post transfection (Fig 3.3 A, lane 4). The decrease in Filamin A levels at day four could not be improved over time, as by day 5 the levels of Filamin A appeared to increase (Fig 3.3 A, lanes 3). The levels of Filamin A in cells transfected with a non-specific scrambled sequence (Ctrl siRNA) remained unchanged (Fig 3.3 A, lane 5-8). SaOS-2 cells transfected with Filamin A-siRNA were subjected to western blot analysis and efficient knockdown of Filamin A (approximately 90%) was observed 5 days post-transfection (Fig 3.3 B, lane 6). The levels of Filamin A in cells transfected with a non-specific scrambled sequence (Ctrl siRNA) remained unchanged (Fig 3.3 B, lanes 1,3 and 5).

Consistent with the decrease of Filamin A protein, RT-qPCR analysis in both cell lines showed a significant decrease of Filamin A mRNA in cells transfected with Filamin A-siRNA in comparison with mRNA levels observed in cells transfected with Ctrl siRNA (Fig 3.3 C).

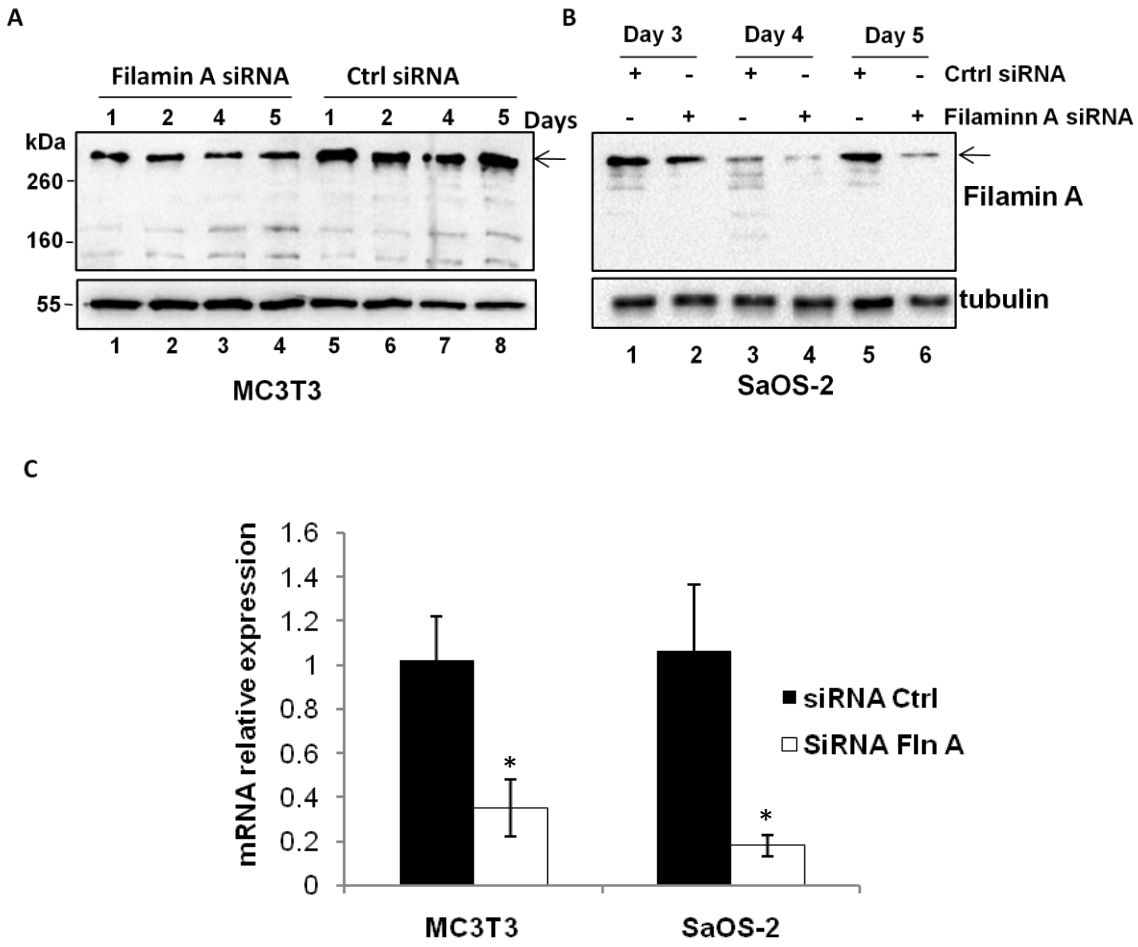


Fig. 3.3 Filamin A knockdown efficiency induced by siRNA. (A) Expression of Filamin A (arrows) was analyzed by western blot in total cell lysates from MC3T3 cells from day one to five after transfection. (B) SaOS-2 cells were transfected with siRNA targeting Filamin A and harvested at day 3, 4 and 5 after transfection; similar results were obtained in three independent experiments. Molecular mass is indicated on the left and tubulin was used as loading control. (C) RT-qPCR analysis shows the reduction in Filamin A mRNA levels when MC3T3 and SaOS-2 were transfected with specific siRNA after day 4 and 5 respectively in comparison with cells transfected with a control siRNA. Data are presented as mean ± standard deviation (S.D.) (n = 3). Statistical evaluation of significant differences was performed using the Student's t-test. Asterisk (*) indicates $P < 0.05$ when compared to control (siRNA control).

3.5 Filamin A does not determine the subnuclear distribution of Runx2.

Bone formation requires transcriptional mechanisms for sequential induction and repression of genes that support the progressive osteoblast phenotype (Lian & Stein, 2003; Lian et al, 2006). Runx2 is important to orchestrate these processes by influencing the functional architecture of target gene promoters (Stein et al, 2000). Furthermore, *in vivo* genetic evidence indicates that interference with subnuclear targeting of Runx2 can block bone formation (Choi et al, 2001). Therefore, since Filamin A is found in the nucleus it could influence the nuclear distribution of Runx2. In order to test this idea, Filamin A-siRNA knockdown was induced in SaOS-2 cells and nuclear Runx2 distribution was analysed by immunofluorescence microscopy. As expected, Filamin A was ablated in cells transfected with Filamin A siRNA in comparison with cells transfected with Control siRNA (Fig. 3.4 A, green panels). Nuclear localisation of Runx2 (red) was detected in Filamin A-siRNA and control siRNA cells (Fig. 3.4 A, Upper and Lower panels, respectively). The established punctate staining pattern of Runx2 was observed under both conditions and no change in nuclear versus cytoplasmic staining was observed. Thus, nuclear localisation of Filamin A does not support efficient nuclear targeting nor subnuclear localisation of Runx2.

Another possibility was that Runx2 could be important for the translocation of Filamin A into the nucleus. Immunofluorescence experiments showed that Filamin A (red) was present in the nucleus of HeLa cells even when this cell type does not express Runx2. Therefore it appears that the subcellular localisation of Filamin A and Runx2 is not inter-dependent.

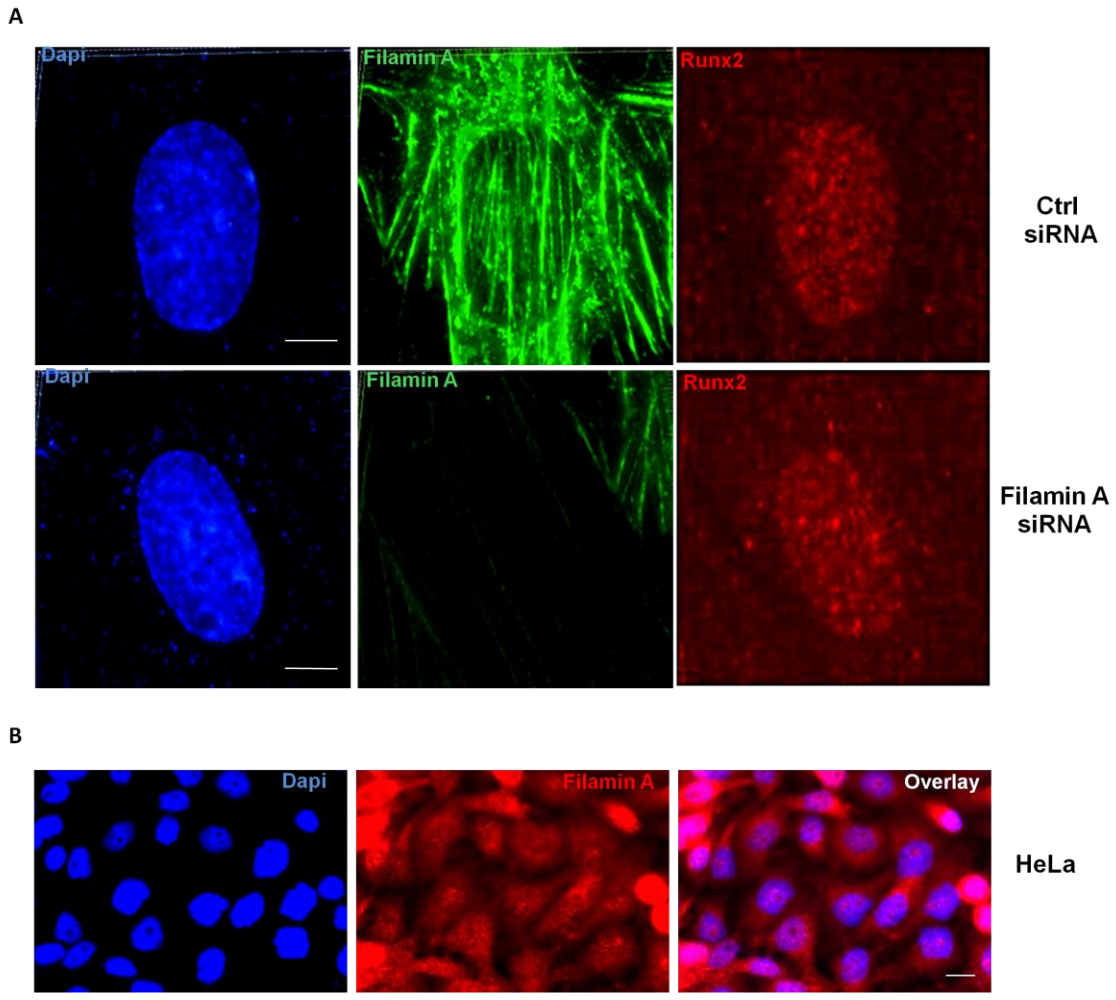


Fig. 3.4 Effect of Filamin A repression on subnuclear localisation of Runx2 protein. (A) SaOS-2 cells were transfected with a Filamin siRNA (upper panels) and a Ctrl siRNA (lower panels) and processed for double immunofluorescence staining. Green and red colour represents Filamin A and Runx2, respectively. Bar, 5 μM. (B) The Runx2-negative cell line HeLa was stained against Filamin A (red) to show that nuclear Filamin A localisation is not dependant on Runx2 expression. Bar, 10 μM.. Blue colour in A and B represents the nucleus stained with Dapi.

3.6 Filamin A affects the cytoplasmic retention of CBFβ but not Runx2

Filamin A functions to retain the Runx cofactor CBFβ in the cytoplasm (Yoshida, 2005). Therefore, as Filamin A and Runx2 interact, Filamin A could possibly retain a portion of Runx2 in the cytoplasm. To test this idea I sought first to reproduce the observation of Yoshida et al, 2005 in the Filamin A-negative (M2) and in the Filamin A-positive (A7) melanoma cell lines. In agreement with Yoshida et al, 2005, immunofluorescence microscopy showed that CBFβ was detected exclusively in the nuclei of Filamin A-negative M2 cells but in the cytoplasm and nucleus of Filamin A-expressing A7 cells (Fig. 3.5 A red and green panels). To confirm the expression of Filamin A in those cells a western blot was performed to show that Filamin A was not present in M2 but in A7. HeLa cells were used as a positive control for Filamin A expression and tubulin was used as a loading control. (Fig. 3.5 B).

After confirming the observation made by Yoshida et al, 2005, the subcellular localisation of Runx2 was determined in the Filamin A-negative M2 and in Filamin A-positive A7 cell lines. As M2 and A7 cells do not express Runx2, Runx2 was expressed by transient transfection. After 48 h, when Runx2 could be detected by western blot (Fig. 3.5 C, lanes 3, 4), cells were processed for immunofluorescence against Runx2 and Filamin A (Fig. 3.5 B, red and green panels). Runx2 was located mainly in the nucleus of the Filamin A-negative as well as in Filamin A-positive cells. These results suggest that Filamin A does not sequestrate Runx2 in the cytoplasm unlike what is the case for its heterodimeric partner CBFβ.

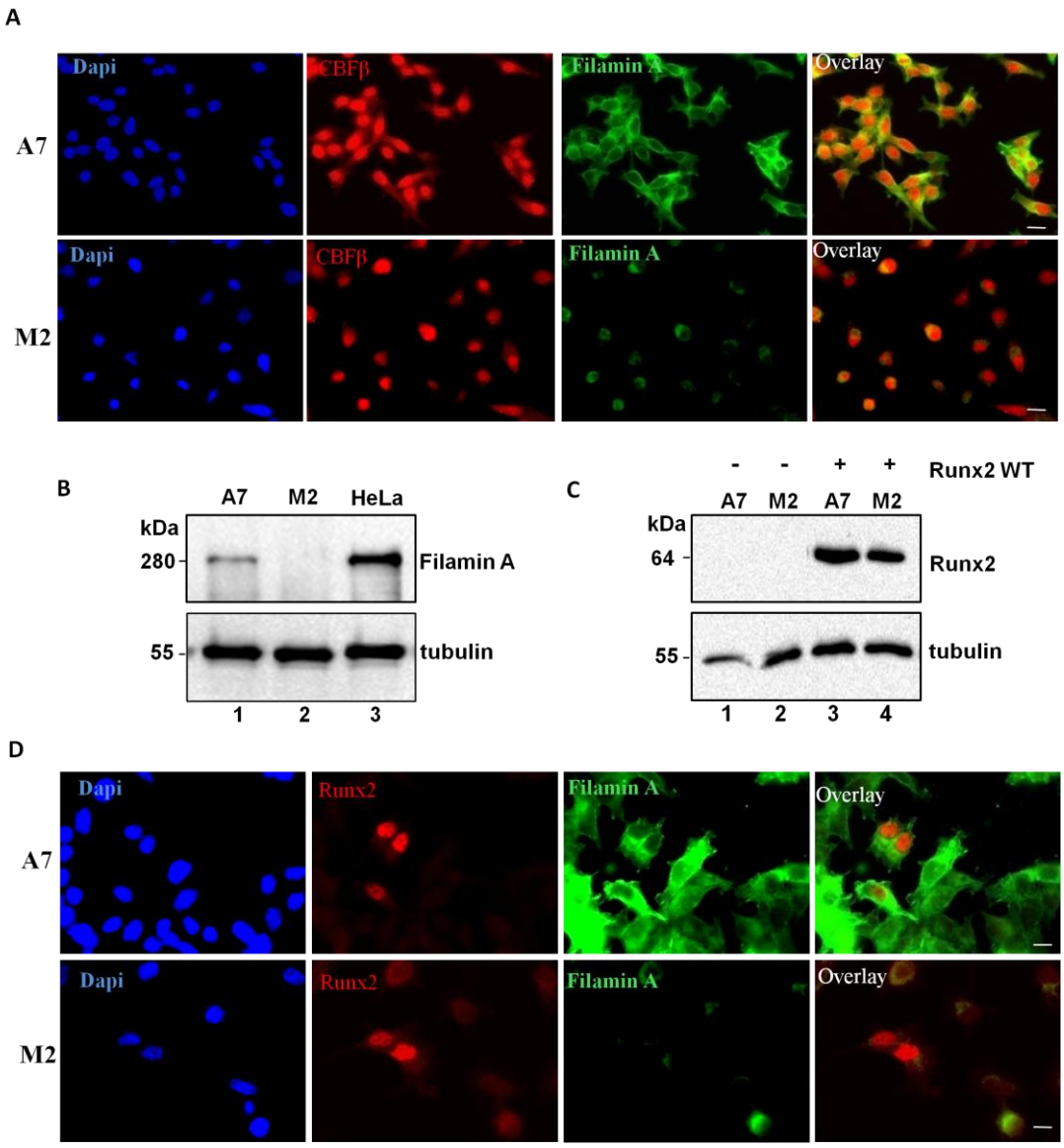


Fig. 3.5 Subcellular localisation of CBFβ and Runx2 in M2 and A7 cells. (A) M2 and A7 immunofluorescence staining against CBFβ (red) and Filamin A (green). (B) Filamin A expression in A7, M2 and HeLa cells by western blot of total cell extracts. (C) Runx2 expression in M2 and A7 cells previously transfected with full-length Runx2 (lanes 3,4) and with control plasmid (lanes 1,2) after 48 hrs of transfection. Molecular mass is indicated on the left and tubulin was used as a loading control. (D) M2 and A7 cells transfected with full-length Runx2 were stained against Runx2 (red) and Filamin A (green). Blue colour represents the nucleus stained with DAPI. Bars, 10 μm.

3.7 The effect of Filamin A downregulation on Runx2 target genes

The results so far in this thesis indicate that Runx2 and Filamin A can form a complex in osteoblastic cells and that the presence or absence of Filamin A does not affect Runx2 subnuclear localisation. In order to determine the functional effect of the Filamin A interaction with Runx2, Runx2-target gene expression was analysed in the presence and absence of Filamin A. The following known Runx2-target genes were analysed: Osteocalcin (OC), Osteonectin (ON), Matrix Metallo proteinase-13 (MMP-13), and Osteopontin (OPN). MC3T3 cells were cultured and transfected twice with Filamin A siRNA or control siRNA in a five day period, resulting in 90% downregulation of Filamin A (Fig. 3.6 A). mRNA from cells was isolated, cDNA synthesised and RT-qPCR was performed. The primers used in this study were designed to amplify around 100 and 120 base pairs for each gene in study and all of them were able to amplify only one PCR product (Fig. 3.6 B). Levels of mRNA for Filamin A decreased in Filamin A-siRNA transfected cells, which correlates with the low amount of Filamin A protein (Fig. 3.6 C and A). The Runx2 target genes OC, OP and ON showed no significant difference when Filamin A was present or absent. However, MMP-13 showed an increase in cells transfected with Filamin A siRNA in comparison with the control. The downregulation of Filamin A did not change the levels of Runx2 nor those of CBFβ, suggesting that the results obtained in this experiment are not due to changes in the amount of Runx2 or CBFβ. Thus, these findings suggest that Filamin A inhibits the expression of the Runx2-target gene *MMP-13*.

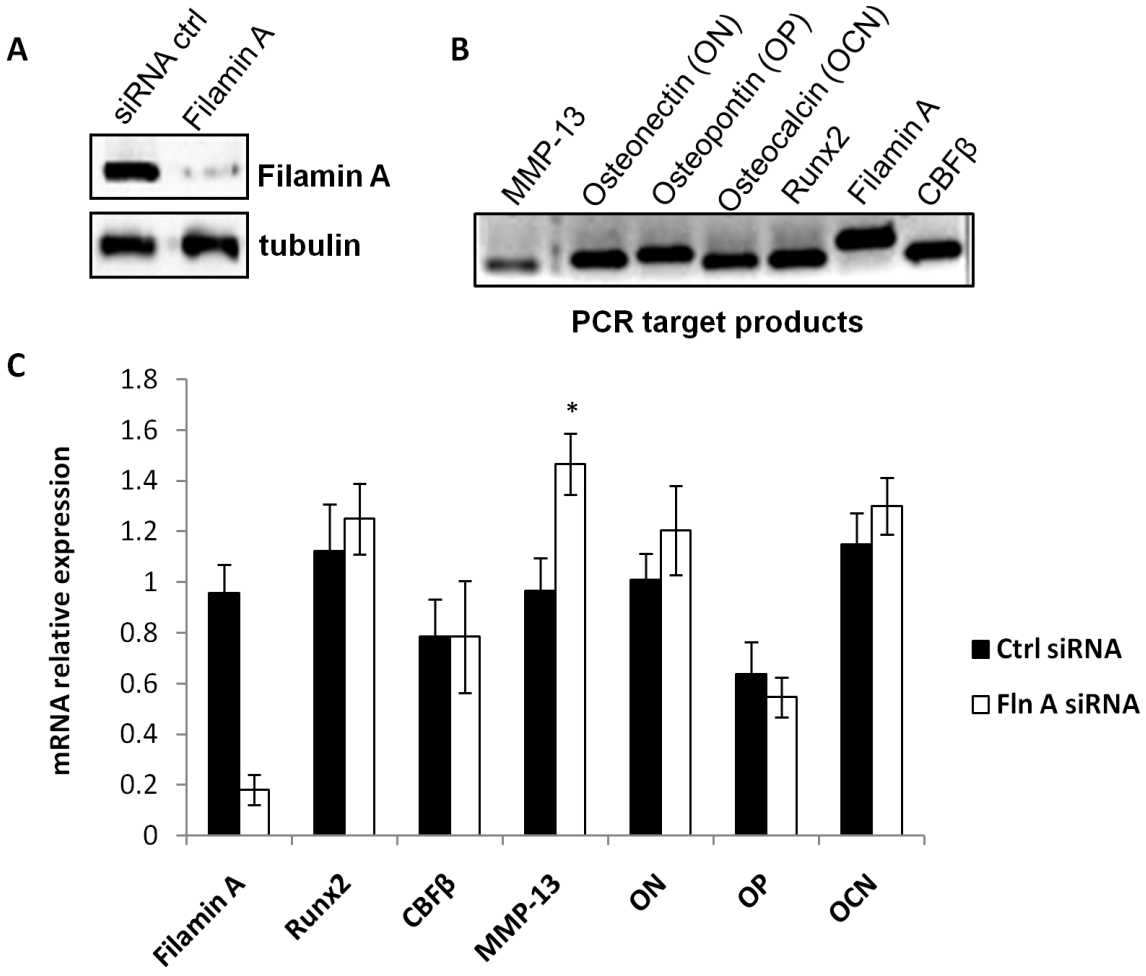


Fig. 3.6 Realtime-qPCR analysis of known Runx2-target genes after siRNA knockdown of Filamin A. (A) A western blot of Filamin A shows Filamin A downregulation in MC3T3 cells after transfection with specific siRNA. (B) The genes examined are indicated on the X-axis which showed one amplification product. (C) RT-qPCR in MC3T3 cells. Cells were transfected with either control (black bars) or Filamin A (white bars) siRNA and processed for qPCR studies. To assess changes in gene expression the $2^{-\Delta\Delta ct}$ method was used. GAPDH was used as a reference housekeeping gene. Statistical evaluation of significant differences was performed using the Student's t-test. Asterisk (*) indicates $P < 0.05$ when compared to control (siRNA control).

3.8 Filamin A suppresses the Runx2-target gene *MMP-13*

In the previous section it was shown that MMP-13 was significantly up-regulated when Filamin A was absent, whilst OCN and OP showed no significant change (Fig. 3.6). This observation suggests that Filamin A could influence specific Runx2 target genes. MMPs are members of a large family of proteinases that have the capacity to cleave native and interstitial collagens (Aimes & Quigley, 1995). Above all the collagenases, MMP-13 (collagenase-3) has been considered to have an important role in skeletal formation as it is present exclusively in the skeleton during embryonic development in cartilaginous growth plates and primary centres of ossification (Mattot et al, 1995). To determine if Filamin A suppresses *MMP-13* gene transcription, the activity of the MMP-13 promoter was analysed using a luciferase promoter reporter in Filamin A knockdown SaOS-2 cells. The human MMP-13 promoter contains two Runx sites (Fig.3.7 A). The MMP-13 reporter plasmid (pGL3MMP-13) used in this study encodes the wild-type sequence and contains the two Runx recognition sites. pGL3MMP-13mut was generated by mutating both Runx-binding sites by site directed mutagenesis (Fig. 3.7 B). Co-transfection of Filamin A siRNA with pGL3MMP-13 resulted in a 2 fold increase in the promoter activity compared to control siRNA transfections (Fig. 3.7 C, white bars). Importantly, when the Runx-binding sites in the MMP-13 promoter were mutated, knockdown of Filamin A had no effect on the activity of the MMP-13 promoter (Fig. 3.7 C, black bars). Therefore, it is suggested that Filamin A suppression of the MMP-13 promoter is mediated via Runx2.

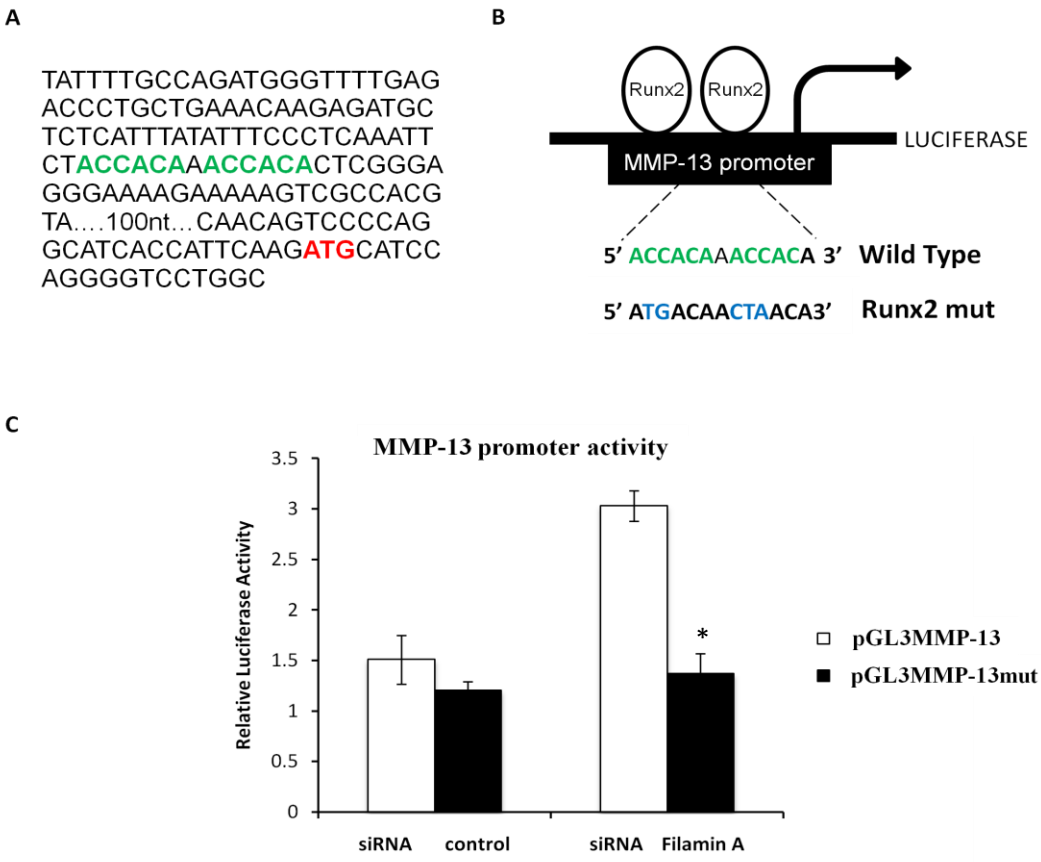


Fig. 3.7 Knockdown of Filamin A activates in a Runx2 dependent manner the MMP-13 promoter activity. (A) Representation of the proximal MMP-13 promoter from human sequence. Runx sites are shown in green, red letters denotes the translational start site. (B) Diagram of pGL3MMP-13 wild type promoter reporter plasmid showing the Runx2 sites in green. The induced Runx2 site mutations in order to produce pGL3MMP-13mut are shown in blue. (C) SaOS-2 cells were transfected with either control or Filamin A siRNA and then transfected with either the wild type pGL3MMP-13 or the pGL3MMP-13mut promoter reporters as indicated. Data are presented as mean ± standard deviation (S.D.) (n = 3). Statistical evaluation of significant differences was performed using the Student's t-test. Asterisk (*) indicates $P < 0.05$ when compared to pGL3MMP-13 in siRNA-Filamin A cells.

3.9 Filamin A suppresses the MMP-13 promoter via Runx2 in M2 and A7 cells

To further establish if *MMP-13* suprression by Filamin A suppression is indeed mediated by Runx2, ectopic expression of Runx2 was induced in Filamin A-negative (M2) and the Filamin A-positive (A7) cell lines were used (Fig. 3.5 and Fig. 3.8 B). M2 and A7 cells do not express Runx2. Therefore, changes observed in the activity of the transfected MMP-13 promoter would be due to the presence or absence of Filamin A under the Runx2 presence. M2 and A7 cells were transfected with Runx2 plasmid or control plasmid as well as the pGL3MMP-13 or the pGL3MMP-13mut promoter reporter plasmids. After 48 h, cells were harvested and processed for luciferase assay and western blot. The luciferase activity analysis was made by calculating the fold change activity when compared to pGL3MMP-13mut reporter plasmid data. By doing this, the results shown represent only the reporter activity that is dependent on Runx2 overexpresion. In the Filamin A-negative M2 cells, ectopic expression of Runx2 (Fig. 3.8 C, lanes 3,4) stimulated MMP-13 promoter activity by more than 2 fold in comparison with the control M2 cells that not received the Runx2 plasmid (Fig. 3.8 A, M2 bars and 3.8 C lanes 1 and 2). This indicates that transfected Runx2 is active and sufficient to induce MMP-13 promoter activity in these cells. In contrast, expression of Runx2 in the Filamin A-expressing A7 cells had no effect on the promoter activity in comparison with A7 cells where Runx2 was not present (Fig. 3.8 A, A7 bars). Furthermore, Runx2 expression in Filamin A-negative M2 cells stimulated the activity of the promoter by more than 2 fold in comparison with the 1.4 fold from the Runx2 transfected Filamin A-expressing A7 cells. Taken together these data indicate that Filamin A-negatively regulates the Runx2-dependent activity of the MMP-13 promoter.

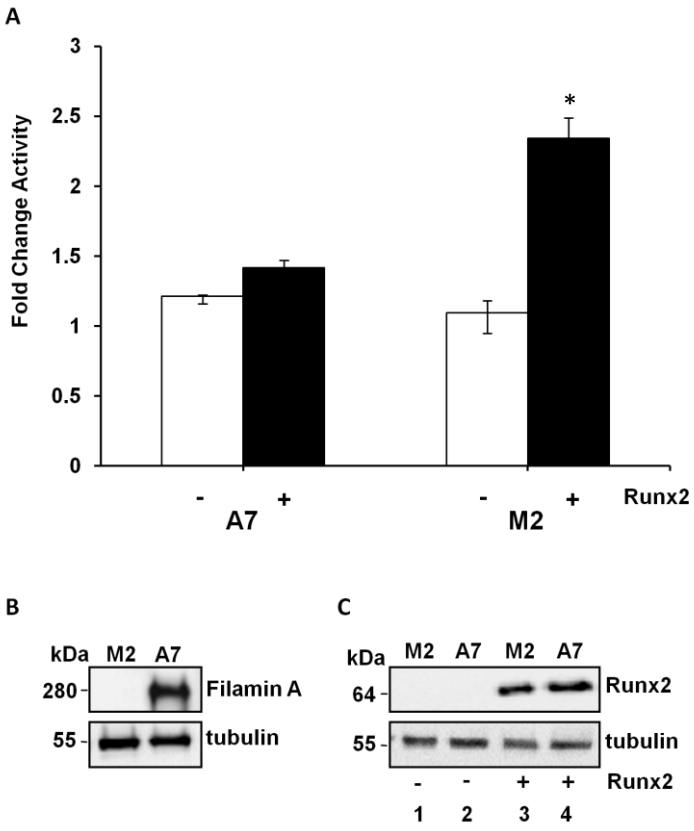


Fig. 3.8 Filamin A suppresses Runx2 activation of the MMP-13 promoter. (A) M2 and A7 cells were transfected with the pGL3MMP-13 promoter reporter in the presence and absence of a Runx2 expression plasmid as indicated. The luciferase assay data show the fold change in activity compared to the activity of the transfected pGL3MMP-13mut Data are presented as mean ± standard deviation (S.D.) (n = 3). Statistical evaluation of significant differences was performed using the Student's t-test. Asterisk (*) indicates $P < 0.05$ when compared to M2 cells transfected with control plasmid. (B) A western blot confirming the Filamin A expression status of M2 and A7 cells. (C) A western blot demonstrating that M2 and A7 cells do not express Runx2 (lanes 1,2). Runx2 was detected after Runx2 plasmid transfection (lane 3,4).

3.10 Filamin A is downregulated during differentiation

It is well established that MMP-13 expression increases during osteoblast differentiation (Mizutani et al, 2001). MC3T3 cells represent a well accepted model for studying osteoblastic differentiation in vitro. The osteoblastic differentiation can be induced by incubating confluent MC3T3 cells with ascorbic acid and β-glycerol phosphate (Franceschi et al, 1994; Harada et al, 1991) It has been observed that differentiating cells start to form a well mineralized extracellular matrix (ECM) from day 4 onwards and by two weeks they form a substantial amount of mineralized deposits, a crucial step to form calcified bone (Mizutani et al, 2001). Also, these cells selectively express mRNAs for the osteoblast markers, MMP-13, bone sialoprotein (BSP), osteocalcin (OCN), and express comparable basal levels of mRNA encoding Runx2 (Bourne et al, 2004). Given the data suggesting that Filamin A suppresses MMP-13 expression, the expression of these proteins during differentiation in MC3T3 cells was determined.

In order to confirm the ability of these cells to differentiate, cells were treated with or without ascorbic acid and β-glycerol phosphate for two weeks. Cells were fixed and incubated with alizarin red which stains for the presence of calcium deposition denotating thus differentiation. Therefore, alizarin staining was used as an early stage marker of matrix mineralisation. As expected, differentiating MC3T3 cells formed mineralized deposits (black patches) in comparison with control cells (Fig. 3.9 A). Next, the expression of Filamin A and MMP-13 in control and differentiating cells by western blot was determined. In agreement with previous reports MMP-13 expression increased significantly from day 4 to day 16 (Fig. 3.9 B, top panel). Surprisingly, Filamin A expression was markedly reduced from day four through to day sixteen (Fig. 3.9, B

middle panel). Moreover, the changes in protein expression of Filamin A and MMP-13 were correlated with changes of mRNA levels, as analysed by RT-qPCR (Fig. 3.9 C). Thus, maximum expression MMP-13 and minimum expression of Filamin A were observed at 16 days. Therefore the expression of endogenous MMP-13 correlates inversely with that of endogenous Filamin A in differentiating osteoblastic cells; supporting the hypothesis that Filamin A suppresses MMP-13 expression.

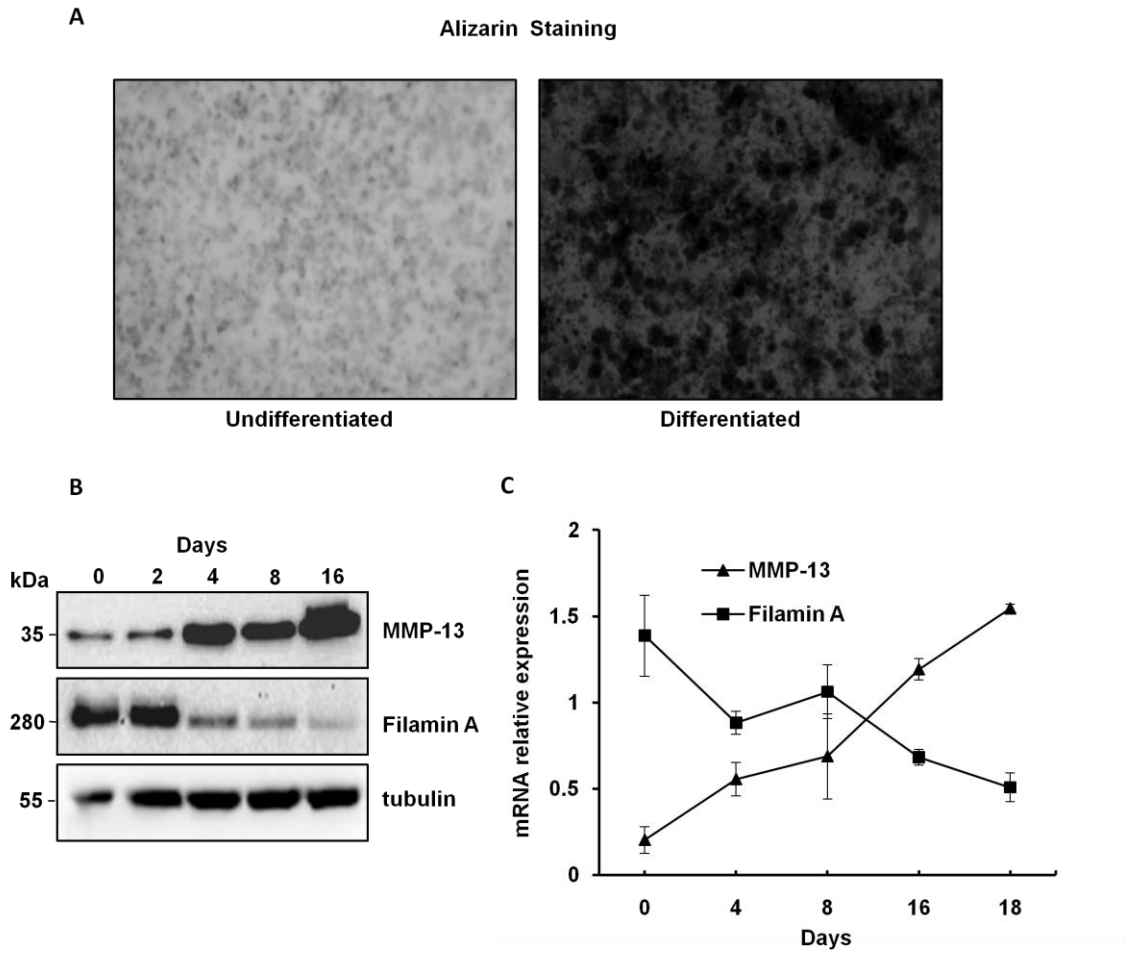


Fig. 3.9 Filamin A is downregulated during differentiation. (A) Mineralization deposits as shown by alizarin red staining were observed after two weeks differentiation period in comparison with differentiated cells. Photos are shown in black and white. (B) Expression of Filamin A and MMP-13 in differentiating MC3T3 cells. Total cell extracts were analyzed by western blotting with antibodies as indicated. Molecular mass is shown at left and tubulin was used a loading control. (C) RT-qPCR showing mRNA expression of Filamin A and MMP-13 in differentiating cells. Error bars represent the standard deviation (SD) of three independent experiments performed in triplicate.

3.11 The role of Filamin A in the regulation of MMP-13 in differentiating MC3T3 cells

In the last section it was shown that Filamin A downregulation during differentiation correlates with the upregulation of MMP-13. However, this correlation does not prove that Filamin A is regulating MMP-13 expression. In order to show that Filamin A had a direct effect on the regulation of MMP-13, an experiment was performed to re-introduce Filamin A in differentiating cells where Filamin A has been naturally downregulated. Differentiating MC3T3 cells at day 8 were transfected with full length Filamin A or plasmid control. After 48 h RNA was isolated and RT-qPCR for detecting MMP-13 mRNA was performed. As expected in undifferentiated cells, when Filamin A was still present (Fig. 3.10 A lane 1), small but detectable levels of MMP-13 mRNA were present (Fig. 3.10 B, white bar). High levels of MMP-13 mRNA were detected when differentiating cells were transfected with a control vector (Fig. 3.10 B, black bar). In contrast, the reintroduction of Filamin A in differentiating cells (Fig. 3.10 A, lane 3) induced a decrease in the MMP-13 mRNA levels in comparison with differentiating cells that received only the empty vector (Fig. 3.10 B, grey bar). These findings suggest that exogenous expression of Filamin A in differentiating MC3T3 cells suppressed the levels of MMP-13 mRNA.

It has been shown that Filamin A is downregulated by day 4 during differentiation of MC3T3 cells. Therefore, early suppression of Filamin A could induce the differentiated cells to produce more MMP-13 mRNA levels. In order to test this idea, MC3T3 cells were transfected with Filamin A siRNA or control siRNA. Once Filamin A downregulation was induced, cells were differentiated for 5 days, RNA was isolated and RT-qPCR was performed to detect Filamin A and MMP-13 mRNA levels.

As it has been shown before in Fig. 3.9, Filamin A mRNA levels dropped gradually during the differentiation time to reach the lowest levels at day five (Fig. 3.10 C red line, squared markers). In the same cells, levels of MMP-13 increased gradually over the differentiation time (black line, squared markers). When Filamin A was suppressed by siRNA from day zero, it could be shown that Filamin A decreased further its levels over the differentiation time (red line, circled markers). Importantly, suppression of Filamin A from day zero resulted in an increase of MMP-13 mRNA levels over the differentiation time (black line, circled markers). These levels were indeed higher when compared to cells where Filamin A levels decreased naturally during the differentiation period.

Taken together the data so far indicate that Filamin A suppresses the expression of MMP-13 and that this suppression is relieved in some extent during differentiation by downregulation of Filamin A.

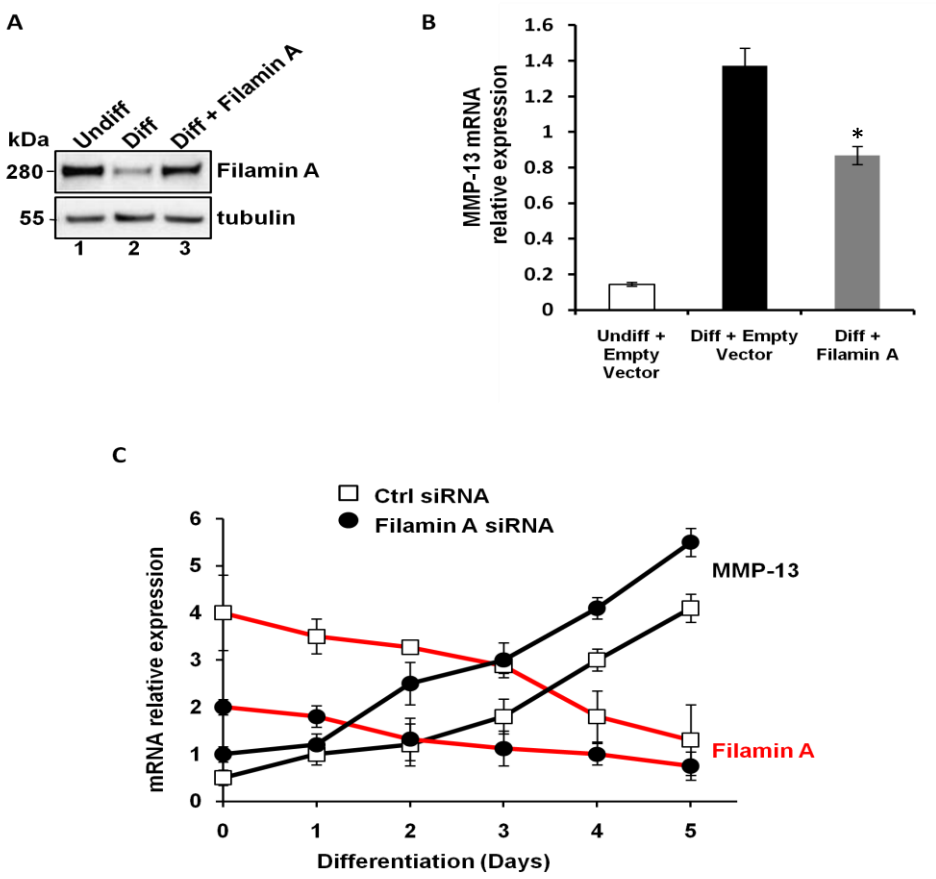


Fig. 3.10 A change in Filamin A levels induces changes in MMP-13 mRNA expression in differentiating MC3T3 cells. (A) A western blot of Filamin A before differentiation, after eight days of differentiation and after exogenous expression of Filamin A in differentiating cells at day eight. Molecular mass is shown at left and tubulin was used as a loading control. (B) RT-qPCR analysis shows that MMP-13 mRNA is reduced after transfection of Filamin A in differentiating MC3T3 cells. Data are presented as mean ± standard deviation (S.D.) (n = 3). Statistical evaluation of significant differences was performed using the Student's t-test. Asterisk (*) indicates $P < 0.05$ when compared to differentiated cells with empty vector. (C) Downregulation of Filamin A by siRNA starting from differentiation day zero induces an increase of MMP-13 levels. Data are presented as mean ± standard deviation (S.D.) (n = 3).

3.12 Filamin A is associated to the MMP-13 promoter

Having demonstrated that Filamin A and Runx2 interact and that Filamin A can suppress the expression of the Runx2-target gene MMP-13 it was next tested if Filamin A was directly involved in the transcriptional regulation of MMP-13. Therefore, it was sought to determine if Filamin A was recruited to the promoter of the MMP-13 gene. Chromatin immunoprecipitation (ChIP) assays were performed in MC3T3 cells. ChIP is a powerful tool for identifying proteins that are associated with DNA by using specific antibodies to immunoprecipitate DNA bound by a protein of interest (Kuo & Allis, 1999). In this experiment MC3T3 cells were subjected to ChIP analysis. Filamin A and Runx2 antibodies were used to pull-down protein-DNA complexes and IgG was used as a control. Endogenous Filamin A and Runx2 were both present on the endogenous MMP-13 promoter in MC3T3 cells when compared to control IgG experiments (Fig. 3.11 A). In contrast, Filamin A nor Runx2 could be detected on the promoter of the osteopontin gene, which was unaffected by knockdown of Filamin A (Fig. 3.6), and is consistent with the fact that osteopontin is expressed at later stages of differentiation (Beck et al, 2000). Furthermore, Filamin A was present at the MMP-13 promoter prior to differentiation but was not detectable after 6 days of differentiation (Fig. 3.11 B). These results show that Filamin A is associated to the MMP-13 promoter and suggest that prior to differentiation of osteoblasts Filamin A is associated to the MMP-13 promoter and during differentiation Filamin A is downregulated, thus relieving suppression of the MMP-13 promoter. Taken together the data presented in this chapter demonstrate that Filamin A interacts with Runx2 to directly suppress Runx2-dependent expression of the MMP-13 gene.

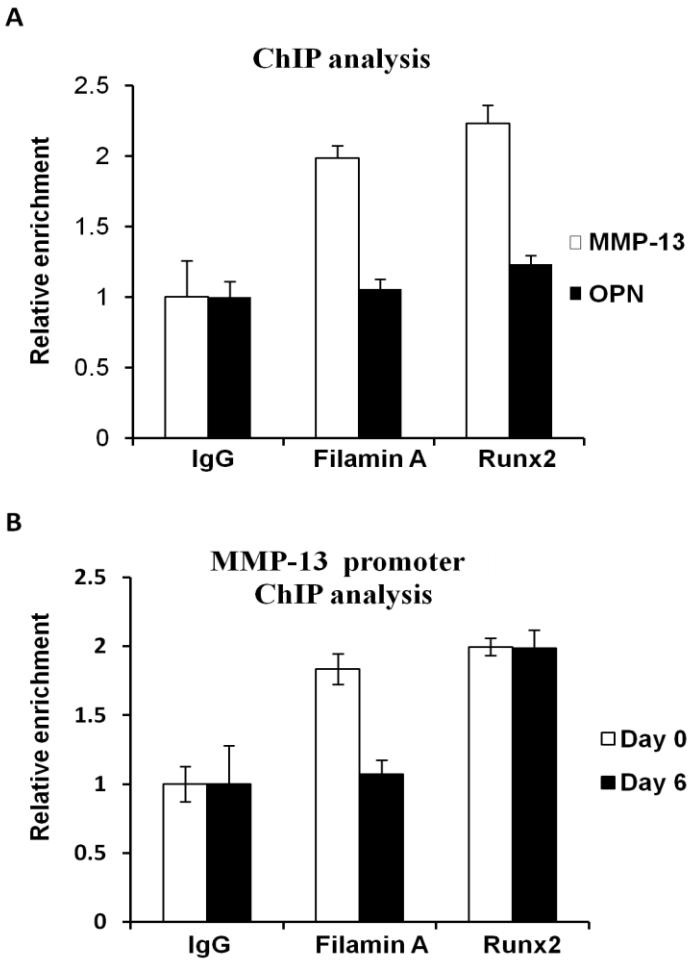


Fig. 3.11 Chromatin immunoprecipitation (ChiP) analysis shows that Filamin A is associated to the MMP-13 promoter. (A) ChIP assays showing Filamin A and Runx2 associated to the MMP-13 promoter. ChIP assays were performed with antibodies as indicated on the X-axis. (B) ChIP assays showing Filamin A is no longer associated to the MMP-13 promoter in differentiating MC3T3 cells. ChIP assays were performed with antibodies as indicated on the X-axis on day 0 and day 6 of differentiation. The data show RT-qPCR performed with MMP-13 and/or OPN promoter primers. Data in A and B are presented as mean ± standard deviation (S.D.) (n = 3).

3.13 Discussion

The data presented in this study demonstrates that Filamin A and Runx2 can interact *in vivo* in osteoblastic cells. It also shows that Filamin A is present in the nucleus and can regulate, via Runx2, the activity of the MMP-13 gene by binding to its promoter. Moreover, it has been demonstrated that during osteoblastic differentiation, Filamin A is downregulated, which in turn promotes the activation of the osteoblastic differentiation marker MMP-13. Variations of Filamin A levels during differentiation can affect the levels of MMP-13, therefore suggesting that presence of Filamin A delays the osteoblastic differentiation process.

3.13.1 The interaction between Filamin A and Runx2

The data presented in this thesis shows for the first time that endogenous Filamin A interacts with the transcription factor Runx2 in osteal cells. Filamin A is an actin-binding protein which organises the actin filaments and functions as a scaffold for a wide variety of proteins, some of them involved in signal transduction pathways (Popowicz, 2006). To date, the transcription factors Insulin-like growth factor-binding protein-5, the androgen receptor, the smads proteins, foxc1 and p73 alpha have been found to be regulated by Filamin A which has positive and negative influences on transcription (Abrass & Hansen, 2010; Berry et al, 2005; Kim et al, 2007; Loy et al, 2003; Sasaki et al, 2001).

Previously, it has been demonstrated that Filamin A binds to and retains the Runx2 binding partner, CBFβ, in the cytoplasm (Yoshida, 2005). One important question to discuss is whether Runx2 is directly binding to Filamin A or if Filamin A is bound to CBFβ and in consequence Runx2 is carried over indirectly. Results gathered in this

regard (Tang Y and Shore P, unpublished data) have demonstrated by *in vitro* GST-pulldowns of purified proteins that sequential deleterious truncations in the carboxy-terminal of Runx2 up to amino acid 424 were able to bind to Filamin A. However, further deletion to amino acid 396 significantly reduced the interaction, as did all other C-terminal deletions up to residue 235. Residues between 396 and 424 of Runx2 were therefore essential for the interaction with Filamin A. With this evidence, as CBFβ was not present in those pulldowns, it can be concluded that the interaction between Filamin A and Runx2 indeed occurs and in this case this interaction can have an important biological function in osteoblastic cells.

3.13.2 Nuclear Filamin A and Runx2 co-exist in the nucleus

Filamin A, besides being a cytoskeletal protein, has also been found in the nucleus (Bedolla et al, 2009; Dingova et al, 2009; Ozanne et al, 2000; Uribe & Jay, 2009; Wang et al, 2007). Reports have suggested that only the cleaved version of Filamin A can be translocated into this organelle. Full length Filamin A is cleaved to a 90 kDa fragment which is then translocated to the nucleus in hormone-naïve cells. Alternatively in hormone-refractory cells, Filamin A was phosphorylated, preventing its cleavage and nuclear translocation (Bedolla et al, 2009). Moreover, it has been reported that a C-terminal 100 kDa fragment of Filamin A colocalised with the Androgen Receptor to the nucleus, but full length Filamin A remained predominantly in the cytoplasm (Loy et al, 2003). Conversely, a small fraction of full length Filamin A has been found to be located in the nucleus of human skin fibroblasts and HeLa cells, where it participates in the DNA damage response through a nuclear interaction with BRCA2 (Yue et al, 2009). Therefore, there is some controversy as to whether full-length Filamin A is present in

the nucleus of cells (Zhou et al, 2010). In this thesis evidence is presented by immunofluorescence and western blot analysis which shows that full length Filamin A is not only present in the cytoplasm but also it is abundantly expressed in the nucleus in osteoblastic cells. These observations have been also extended to other cell-types (HeLa, PC3, C2C12 and U2-OS) but not in the LNCaP cell line, where nuclear Filamin A is not detected (Wang et al, 2007). These findings suggest the possibility that nuclear Filamin A can affect the function of the transcription factor Runx2. Therefore, as both proteins have been shown to interact *in vivo*, it is likely that this interaction occurs in the nucleus.

3.13.3 Filamin A binds to the MMP-13 promoter to suppress gene expression via Runx2

Filamin A interacts directly with several diverse proteins including receptors, kinases and transcription factors and has been proposed to act as a scaffold for signalling transduction pathways. Since 2000, reports have been published regarding the interaction between Filamin A and transcription factors (Popowicz, 2006). It has been demonstrated that the regulation of the transcription factor called FOXC1 is mediated through many interactions with both Filamin A and the transcription regulator PBX1. The full length Filamin A efficiently carries PBX1 into the nucleus where the FOXC1-PBX1 transcription-inhibitory complex forms (Berry et al, 2005). Moreover, the nuclear presence of the Filamin A fragment interferes with the interdomain interactions and co-activator binding of another transcription factor, the Androgen Receptor, thus repressing its transactivation activity (Loy et al, 2003). In this work it has been demonstrated that Filamin A suppresses Runx2-dependent expression of MMP-13 in osteoblasts. These

findings provide the first molecular explanation of how Filamin A modulates bone-specific gene expression. Also it has been shown that Filamin A binds directly to Runx2 and both proteins are associated to the MMP-13 promoter. Little is known about the nuclear function of Filamin A, although it has been shown to inhibit the activity of the transcription factors FOXC1 (Berry et al, 2005) and the Androgen Receptor (Loy et al, 2003) and, with this thesis, it can be added Runx2 to the list. Currently, Filamin A is thought to be present in the cytoplasm and in the nuclear fraction, which can suggest that Filamin A can have distinct functions apart from being an actin binding protein. The association of transcription factors with this cytoskeletal protein, either the full length or the cleaved product, is particularly interesting as it suggests that Filamin A plays a pivotal role in linking the cytoskeleton to the regulation of gene expression. Whether Filamin A can affect a different set of transcription factors, either by cytoplasmic sequestering or by influencing its nuclear activity, is still unclear and remains to be a matter of subsequent studies.

3.13.4 The downregulation of Filamin A and the upregulation of MMP-13 in differentiating osteoblasts

Besides the typical function of Filamin A as an actin cross-linking protein, or as a scaffold for cytoplasmic signalling proteins. Filamin A plays essential roles throughout development and in the adult organism. Mutations in each of the human Filamin genes have been linked to disease with similar phenotypes including embryonic lethality, defective neuronal migration, valvular dystrophy, congenital bone malformations, and myofibrillar myopathy (Zhou et al, 2010). This diversity reveals that Filamins perform a variety of essential functions, particularly with respect to the skeletal and

cardiovascular systems. One important finding in this work is the fact that during the osteoblastic differentiation of MC3T3 cells the levels of Filamin A are downregulated from day 3 onwards, which coincides with the upregulation of MMP-13 protein, a marker of osteoblastic differentiation (Zaragoza et al, 2006). Furthermore, Filamin has been shown to be downregulated during differentiation of different lineages, this is an interesting observation that led to think of Filamins as regulators of differentiation during development and in adult organisms. Filamin downregulation during differentiation has been reported in the myoblastic cell line C2C12; in contrast, the expression of β1D and sarcomeric myosin heavy chain (MHC) was induced in differentiating C2C12 cells as a control for differentiation (van der Flier et al, 2002). Also, it has been reported that the induced differentiation with retinoic acid in the promyelocytic NB4 and myeloblastic PLB985 cells towards the granulocytic pathway induced the downregulation of Filamin A and the upregulation of the ASB2 which inhibits growth and promotes cell fate commitment. ASB2 is the specificity subunit of an E3 ubiquitin ligase complex and is proposed to exert its effects by regulating the turnover of specific proteins and in this case targeting Filamin A for proteasomal degradation (Heuze et al, 2008). In this work, the downregulation of Filamin A in differentiating MC3T3 began at day three, and by day four, a considerable amount of Filamin A is decreased in comparison with the control cells. This rapid downregulation might be due to the proteasomal degradation of Filamin A proposed by Heuze et al, 2008. In addition, during myogenic differentiation it has been reported that Filamin A degradation is also favoured by m-calpain which cleaves Filamin A to smaller fragments (Kwak et al, 1993; van der Flier et al, 2002). Therefore it could be that

calpain also has an important role in osteoblastic differentiation by cleaving Filamin A down in order to decrease Filamin A levels. Finally, the levels of the differentiation marker MMP-13 during differentiation was investigated by either expressing or knocking down Filamin A in MC3T3 cells. Thus, Filamin A over expression delayed expression of MMP-13 and Filamin A downregulation favoured its expression. The results presented here point out the importance of Filamin A during osteoblastic differentiation.

3.13.5 A possible mechanism of the regulation of *MMP-13* gene by Filamin A in differentiating osteoblasts.

Given that Filamin A has been shown in this work to be located in the nucleus, to interact with Runx2, to be present in the promoter of the gene *MMP-13* negatively regulating MMP-13 expression, and importantly, to be downregulated during early osteoblast differentiation which coincides with the upregulation of MMP-13; a model of Filamin A function in differentiating osteoblast is suggested (Fig. 3.12). In this model it is proposed that in undifferentiated MC3T3 osteoblasts, when Filamin A levels are unchanged or basal, MMP-13 expression is suppressed in part because Filamin A and Runx2 are associated to the promoter of the *MMP-13* gene (Fig. 3.12). When the MC3T3 cells start to differentiate into osteoblast by adding 10 mM β-glycerol phosphate and 50 µg/ml L-ascorbic acid to the to the growth media, Filamin A expression is subsequently down-regulated (either by calpain cleavage or by proteasomal degradation) by day three. The downregulation of Filamin A results in loss of Filamin A at the MMP-13 promoter so it can no longer suppress Runx2 activity which in turn allows a concomitant increase in MMP-13 expression (Fig. 3.12).

The mechanism of how Filamin A suppresses the activity of Runx2 remains to be elucidated. One mechanism could be that cytoplasmic Filamin A may be an anchor for proteins involved in transcription. Once in the nucleus, Filamin A can bring a specific set of repressors to the promoter, therefore inhibiting the expression of the gene. Another possibility could be that Filamin A and/or Runx2 could promote gene modifications which in turn can block transcription. In terms of epigenetic gene regulation, a gene needs to be activated and chemical labels in the DNA determine which genes are transcribed into RNA and which others will be silenced. Among the epigenetic mechanisms, the silencing of genes by methylation is one of them. This is performed by methyltransferases, enzymes that attach methyl labels to a gene, thus blocking its expression (Zaidi et al, 2010). Further studies will be needed in order to elucidate the mechanism in which Filamin A is suppressing gene expression. The diagrammatic model presented in this thesis is also supported by the fact that mutations in Filamin A, Runx2 or MMP-13 genes cause skeletal dysplasia. It could be possible that mutations in Filamin A give rise to skeletal dysplasia, at least in part, by perturbing the Filamin A/Runx2 regulatory mechanism and thus affecting the expression of MMP-13, and probably other genes that remain to be identified.

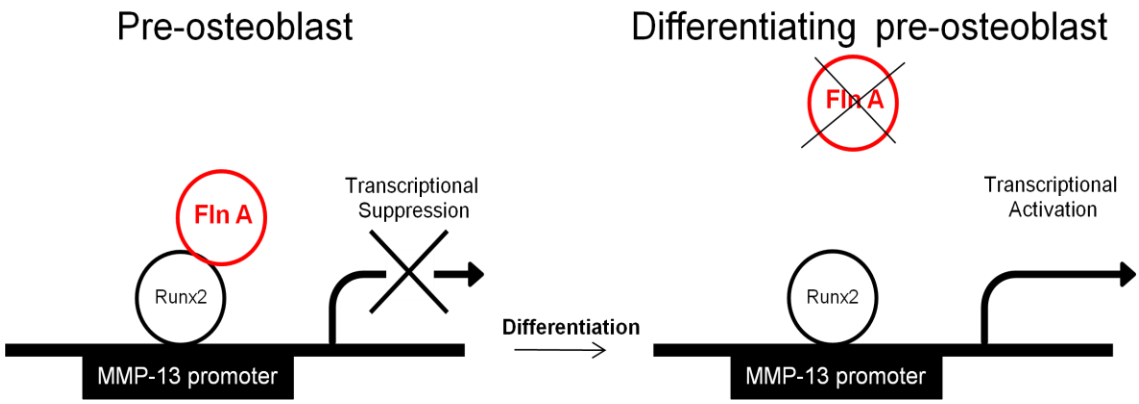


Fig. 3.12 A model depicting control of MMP-13 transcription by Filamin A and Runx2. Filamin A is located in the promoter of MMP-13 to suppress Runx2 function in the pre-osteoblast. During differentiation, Filamin A levels decrease and no longer can inhibit Runx2, thus relieving MMP-13 suppression to continue the osteoblastic differentiation program.

4.0. A novel role of the cytoskeletal protein Filamin A in the nucleolus: the regulation of ribosomal DNA

4.1 Introduction

The essential role of Runx2 in bone development has been demonstrated when mice lacking the Runx2 gene do not form calcified bone (Komori et al, 1997). In addition, Filamin A has been linked to bone malformation in humans. Thus, Filamin A could play a pivotal role in bone formation during development (Robertson et al, 2003; Zhou et al, 2010). The last chapter demonstrated that Filamin A protein is not only able to localise to the cytoplasmic compartment, where it is known to associate with actin, but also that it is abundant in the nucleus. In this chapter it is demonstrated that in the nucleus Filamin A accumulates within the nucleolus in different cell types. The nucleolus is the most prominent nuclear sub-compartment, which has an important role in ribosome biogenesis (Sirri et al, 2008). The nucleolus contains ribosomal genes, rRNA and nucleolar proteins; it is a factory in which rRNAs are synthesized, processed and assembled with ribosomal proteins in order to form pre-ribosome subunits. These subunits travel to the cytoplasm where they direct protein synthesis (Boisvert et al, 2007). Immunofluorescence and co-localisation analysis against distinctive nucleolar proteins suggest that Filamin A is located in the granular component and, in some extent, to the fibrillar centre of the nucleolus. Also, it is shown that Filamin A and Runx2 are found in the nucleolus, in which Runx2 has been previously shown to act as repressor or rDNA genes (Young et al, 2007). Further analyses using a rDNA promoter demonstrated that the Filamin A/Runx2 complex represses ribosomal gene expression and that Filamin A associates with the promoter of ribosomal genes. This chapter presents novel evidence of Filamin A being present in the nucleolus to regulate rRNA expression.

4.2 Punctate patterns of Filamin A are detected in the nucleus

In the previous chapter it was shown that Filamin A is found in the nucleus of the MC3T3 and SaOS-2 cell lines. To further study the subnuclear localisation of Filamin A, immunofluorescence analysis was performed using a different antibodiy raised against a different Filamin A epitope. The EP2405Y antibody showed clear differential staining inside the nucleus (Fig. 4.0 A). These data suggested that nuclear Filamin A was compartmentalized within the nucleus. Subsequent staining of isolated nuclei clearly demonstrated that Filamin A accumulates in discrete bodies within the nucleus. By using the anti-Filamin A antibody "EP2405Y", Filamin A was detected in 5 to 8 punctate accumulations distributed throughout the nucleus in SaOS-2 and MC3T3 cells (Fig. 4.0 B and C lower panels). This observation was consistent by using the anti-Filamin A antibody "ab5127" (Fig. 4.0 B and C upper panels). The circled shaped accumulations seen in each nucleus resembled the size and nuclear distribution of the nucleolus.

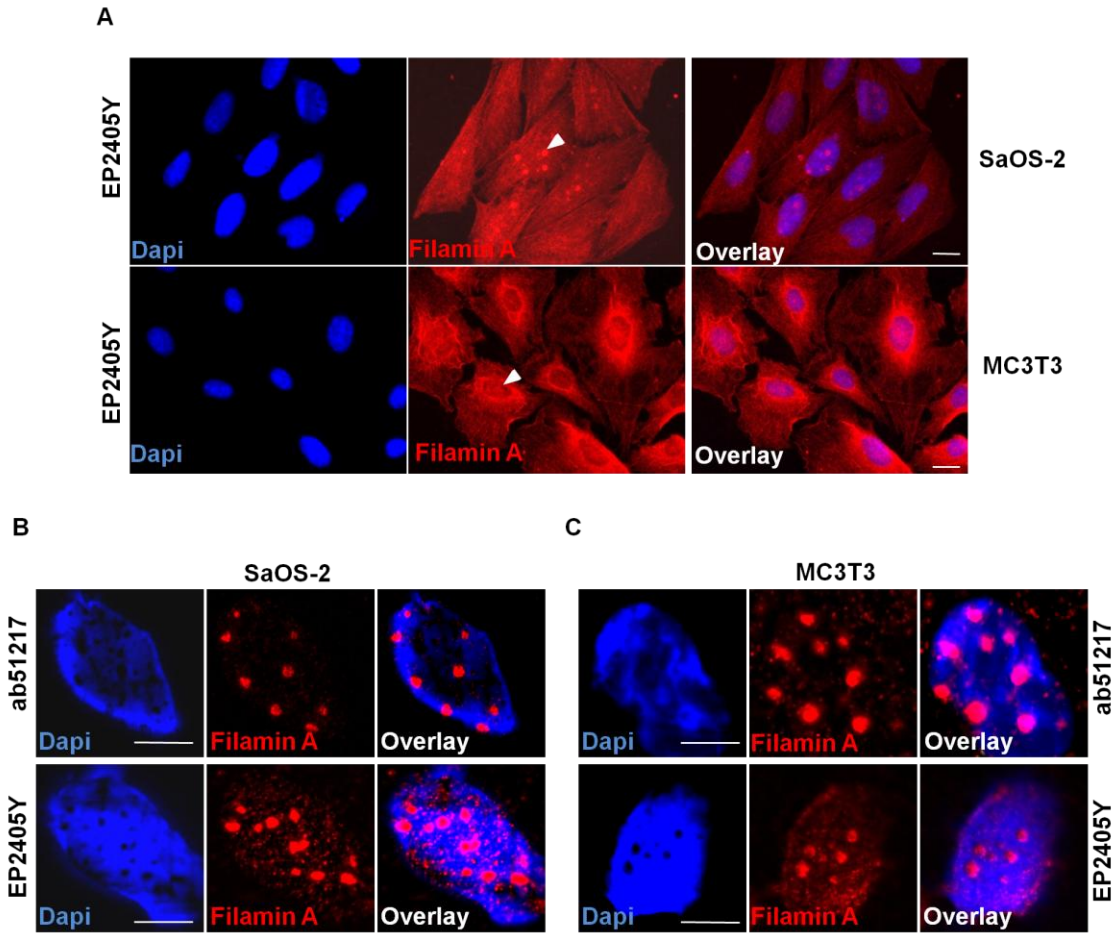


Fig. 4.0 Nuclear Filamin A is located in a punctate pattern in osteoblastic cell lines. (A) SaOS-2 and MC3T3 were immunostained with an anti-Filamin A antibody White arrows show punctate distribution of Filamin A. (B,C) Nuclei isolated from SaOS-2 and MC3T3 cells were stained for Filamin A with two different anti-Filamin A antibodies (as indicated) to show staining similar to nucleolar compartment staining. DNA is stained in blue (Dapi). Bars, 10 μm.

4.3 The Cytoskeletal protein Filamin A is found in the nucleolus

In this work Filamin A has been shown to be express in the nucleus of osteoblastic cells with a distribution similar to that of nucleoli. To determine whether the discrete nuclear bodies containing Filamin A are indeed nucleoli, cells were co-stained for Filamin A and fibrillarin, a well established nucleolar marker (Chen & Huang, 2001). Although small amounts of fibrillarin are found in the cytoplasm it is almost entirely restricted to the dense fibrillar component of the nucleolus (Boisvert et al, 2007; Chen & Huang, 2001; Tollervey & Hurt, 1990).

Co-immunofluorescence staining for Filamin A and fibrillarin in SaOS-2 and HeLa cells demonstrated that Filamin A is located in the nucleolus (Fig. 4.1 A). Nucleolar distribution was also detected in primary cultures of mouse bone marrow stromal cells (Fig. 4.1 B; upper panel shows an apical section against Filamin A staining as indicated by yellow arrowhead). An ecuatorial image of the nucleus in the same specimen showed positive staining in the nucleolar compartment (Fig. 4.1 B, white arrowhead). To corroborate the previous observation, confocal microscopy was performed in isolated nuclei from SaOS-2 cells to show that Filamin A is associated with nucleolar structures (Fig. 4.1 C).

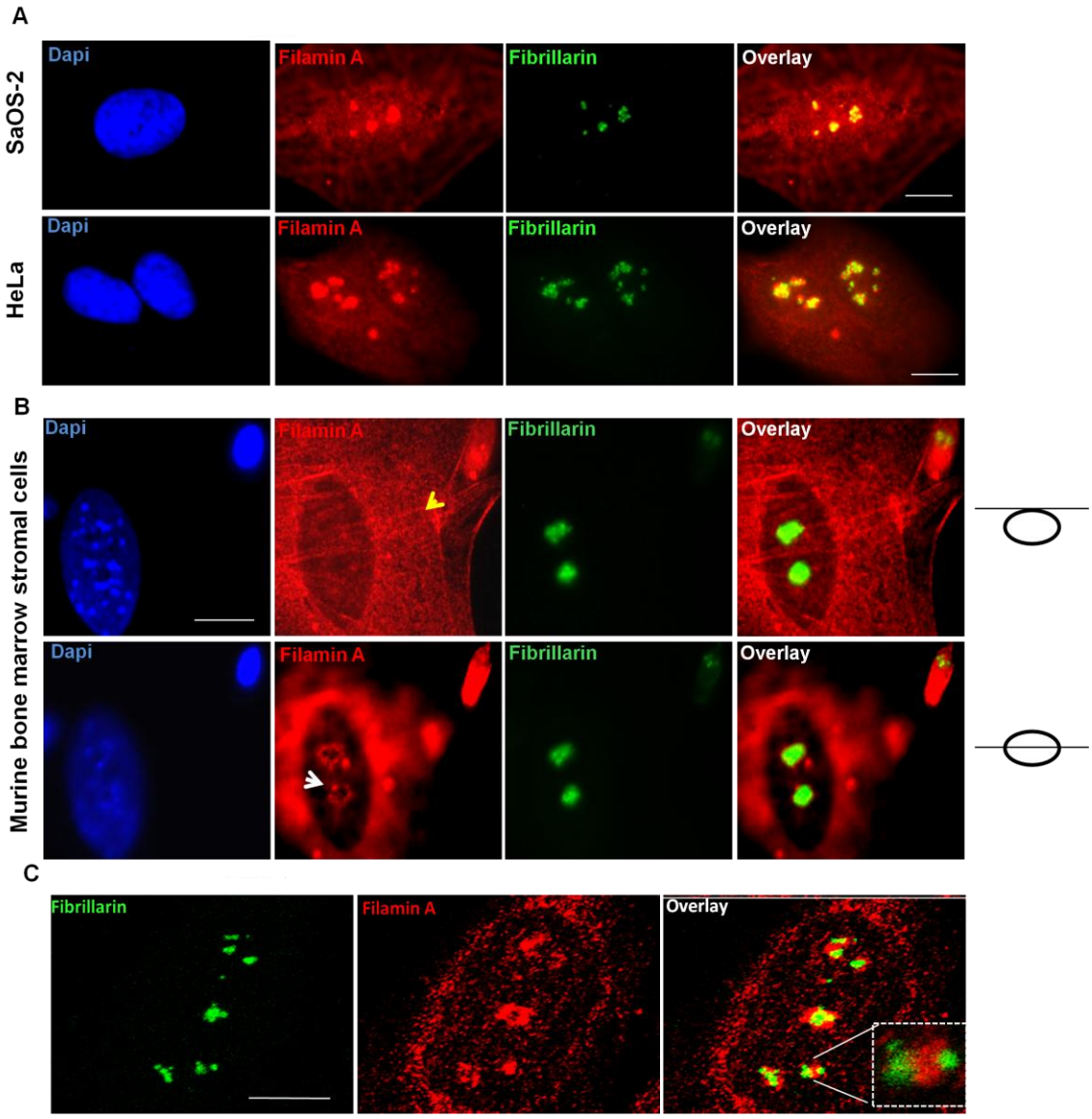


Fig. 4.1 Filamin A localises to the nucleolus. (A) Indirect immunofluorescence analysis of endogenous Filamin A (red) in SaOS-2 (upper panels) and HeLa cells (lower panels) and in murine bone marrow stromal cells (B), as shown co-stained with the nucleolar marker fibrillarin (green). Nuclei (blue) were stained with DAPI. (C) Confocal microscopy in SaOS -2 demonstrates that Filamin A (red) is colocalised to the nucleolus in close proximity with the nucleolar marker fibrillarin (green). Bars, 10 μm.

To confirm the nucleolar localisation of Filamin A, protein components from SaOS-2 were separated into nuclear and nucleolar fractions and subjected to immunofluorescence and western blot analysis. Fluorescence microscopy against fibrillarin (green) in highly purified nucleoli (Ahmad et al, 2009) showed intact isolated nucleoli (Fig. 4.2 A). It can be observed that nucleoli were not degraded or distorted during the isolation method. In agreement with previous observations from intact isolated nuclei, Filamin A (red) was located in the nucleolus (Fig. 4.2 A).

In addition, immunoblot analysis of the subcellular fractions confirmed that Filamin A was within the nucleolar fractions of SaOS-2 and HeLa cells (Fig. 4.2 B). The quality of the nucleolar fractionation was assessed by incubating the blots with antibodies against the cytoplasmic protein tubulin and the nucleolar protein fibrillarin (Fig. 4.2 B middle and lower panels, respectively). In SaOS-2 fractionations, tubulin was detected in the total and nucleolar fraction but not in the nuclear fraction. In HeLa cells, tubulin was detected only in the total fraction (lower panels).

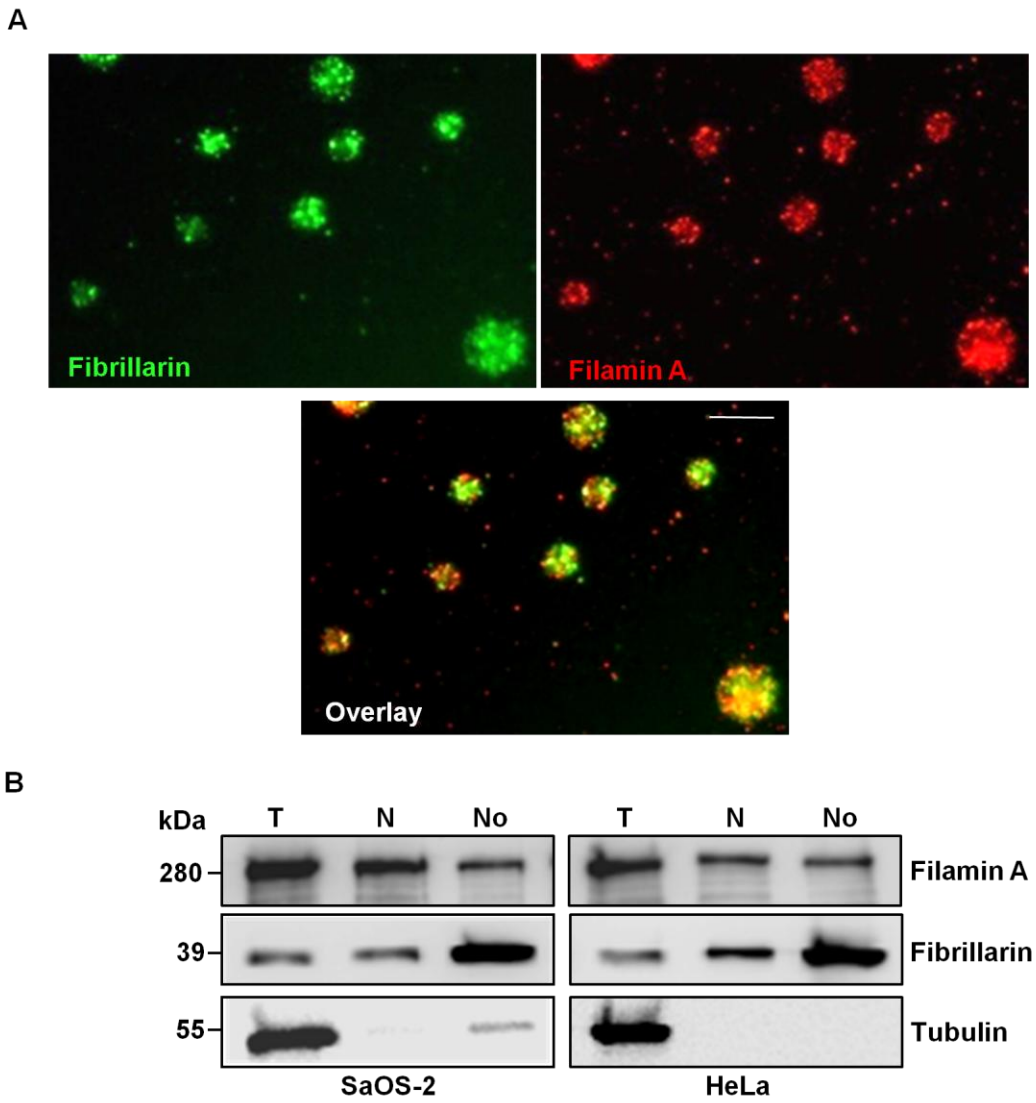


Fig. 4.2 Purified nucleoli contain Filamin A. (A) Highly purified nucleoli from SaOS-2 cells were stained with antibodies against the nucleolar marker fibrillarin (green) and Filamin A (red). Bars, 2 μm. (B) Western blot analysis of Total (T) Nuclear (N) and Nucleolar (No) fractions tested against Filamin, Fibrillarin and tubulin in SaOS-2 (left) and HeLa cells. Molecular mass is shown at left. Tubulin and fibrillarin were used to verify the purity of the cell extracts.

The human melanoma cell line A7, but not M2, expresses Filamin A (Cunningham et al, 1992). Therefore, I decided to establish the subcellular localisation of Filamin A in A7 cells, using M2 cells as the negative control. I confirmed by immunofluorescence that Filamin A could be detected in A7 cells but not in M2 cells; where only a weak background signal was present (Fig. 4.3 A). Filamin A was detected by western blot in the total, nuclear and nucleolar fractionations in A7, but not in M2 cells (Fig. 4.3 B). Fibrillarin and tubulin were used to show the purity of the fractionations. Highly purified nucleoli from A7 and M2 cells was also subjected to double immunostaining for Filamin A (green) and fibrillarin (red) followed by confocal microscopy (Fig. 4.3 C). In agreement with previous observations in other cell types (SaOS-2, HeLa and MC3T3) Filamin A was consistently located in the nucleolus of A7 cells but not in M2 cells.

Taken together the results shown in this chapter give strong evidence that suggests that Filamin A is not only cytoskeletal or nuclear, but also could be an active component of the nucleolus.

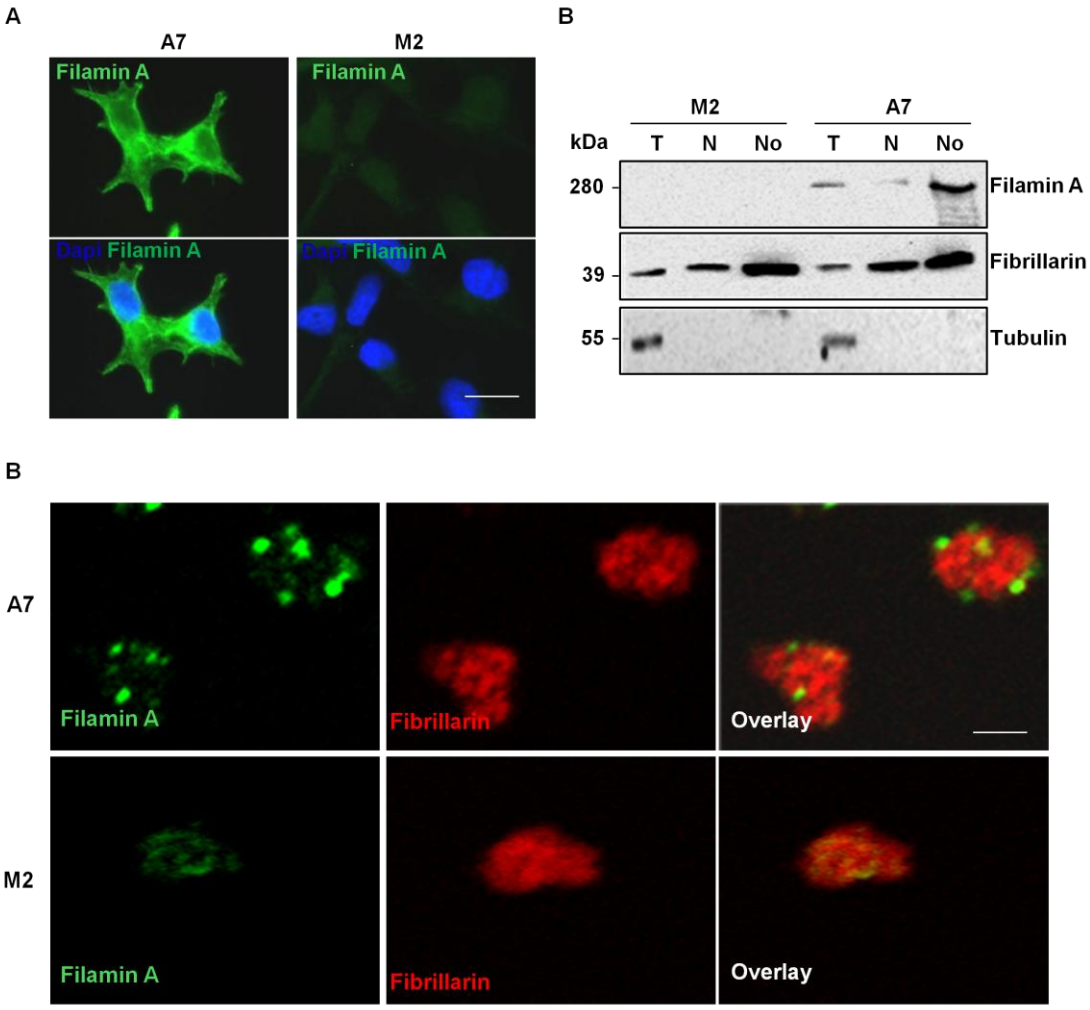


Fig. 4.3 Distribution of Filamin A in isolated nucleoli in A7 cells. (A) M2 and A7 cells immunofluorescence staining against Filamin A. Nuclei (blue) were stained with DAPI. Bar, 20 μm. (B) Western blot analysis of Total (T) Nuclear (N) and Nucleolar (No) fractions tested against Filamin, fibrillarin and tubulin as indicated in M2 and A7 cells. Molecular mass is presented on the left. (C) Confocal analysis of highly purified nucleoli from A7 and M2 cells stained with antibodies against the nucleolar marker fibrillarin (green) and Filamin A (red). Bar, 1 μm.

4.4 Mapping the location of Filamin A in the nucleolus

Having established that Filamin A is a nucleolar protein I next sought to establish whether it localised to a particular sub-nucleloar compartment. In the nucleolus of interphase cells, three morphologically different nucleolar components have been described: the fibrillar centre (FC), the dense fibrillar component (DFC) and the granular component (GC) (Hernandez-Verdun & Louvet, 2004). Transcription of the rDNA is driven between the FC and DFC and most proteins concentrate in the GC region where ribosome subunit assembly is completed. Monoclonal antibodies against nucleolar proteins known to be differentially located in the three different nucleolar compartments (Sirri et al, 2008) were used to determine the sub-nucleolar localisation of Filamin A. The Upstream Binding Factor 1 (UBF-1) and RPA40 are both located in the FC, Nopp140 and fibrillarin are both located in the DFC, and Nucleophosmin (NPM) is located in the GC. Double staining against UBF-1 and Filamin A did not show evident colocalisation between those proteins. (Fig. 4.4 A). However, Filamin A showed partial colocalisation in the FC periphery where RPA40 is located (Fig. 4.4 B). Colocalisation with Filamin A was not detected when stained with fibrillarin or Nopp140 both located in the DFC (Fig. 4.4 C-D, respectively). Staining against NPM and Filamin A showed evident colocalisation in the GC (Fig. 4.4 E).

The sub-nucleolar fluorescence data gathered from this study are summarised in Fig. 4.5 which represents the nucleolar localisation of Filamin A. Filamin A is located primarily within the GC and, to a lesser extent, within the FC. Filamin A was not observed in the DFC.

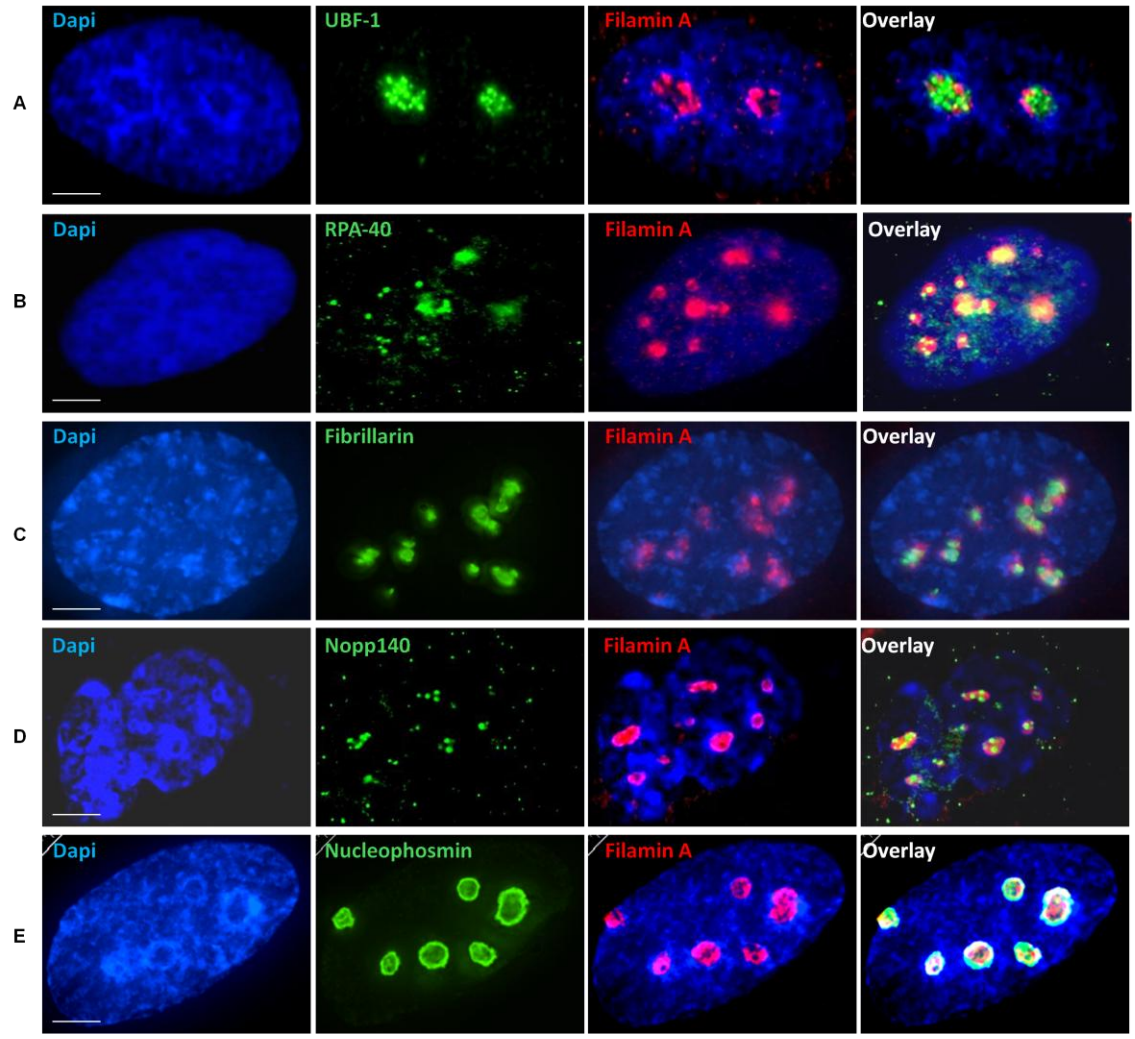


Fig. 4.4 Nucleolar localisation of Filamin A. Confocal laser scanning microscopy of SaOS-2 cells labelled with antibodies against Filamin A and the nucleolar proteins UBF-1, RPA40, fibrillarin, Nopp140- and Nucleophosmin. Nuclei (blue) were stained with DAPI. Bars, 2 µm.

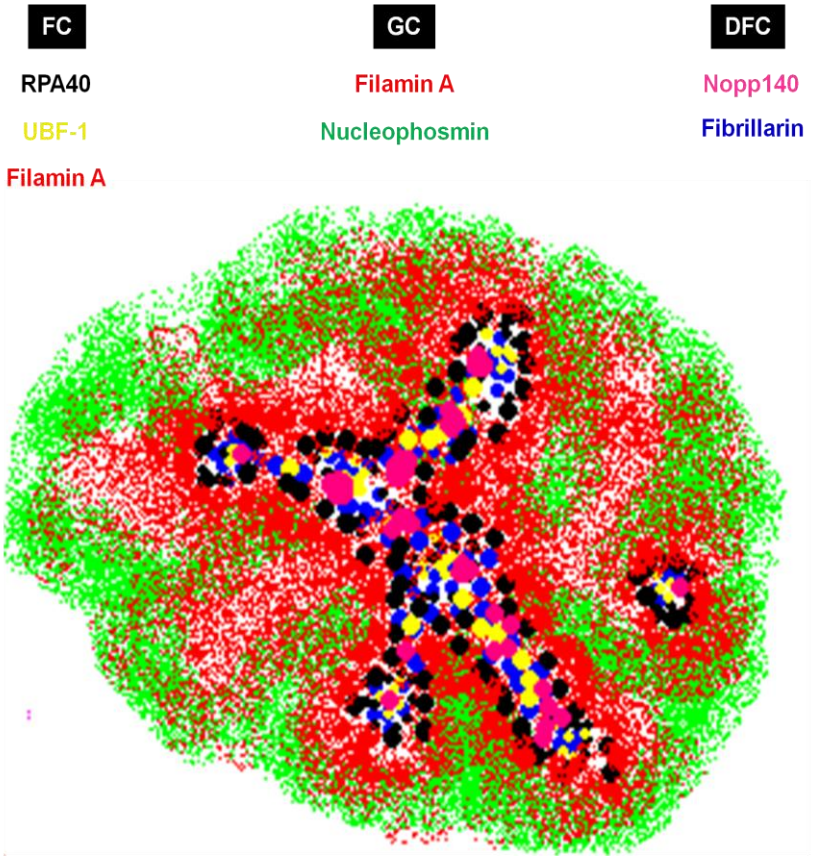


Fig. 4.5 A model representation of the localisation of Filamin A in the nucleolus. The model was made based on confocal analysis data by mapping the localisation of several nucleolar proteins and Filamin A. The Fibrillar centre (FC), the granular component (GC) and the dense fibrillar component (DFC) are represented for the proteins shown in the diagram. Each protein is represented in different colours as stated.

The nucleolus is the place where ribosomal DNA transcription is performed through the activity of RNA Polymerase I. Normally, nucleolar retention of regulatory factors depends on ongoing transcription of rDNA (Grummt, 2003). To examine whether nucleolar localisation of Filamin A depends on ongoing rDNA transcription, I analysed its localisation in the presence or absence of low concentrations of actinomycin D (AMD), which specifically inhibits RNA Pol I transcription by intercalating to the GpC sites of ribosomal DNA (Hadjiolova et al, 1995; Perry & Kelley, 1970). AMD treatment results in the segregation of nucleolar components to the nucleoplasm and their accumulation in the nucleolar periphery (Puvion-Dutilleul et al, 1997; Scheer & Benavente, 1990). SaOS-2 cells were incubated with 50 ng/ml of AMD to completely block rDNA transcription and immunostaining was performed against nucleophosmin and Filamin A (Fig. 4.6). Immunofluorescence analysis of cells treated with AMD showed a segregation of the nucleolar protein nucleophosmin (NPM) throughout the nucleoplasm and the previously reported "cap shape" (white arrow) in the nucleolar periphery (Shav-Tal et al, 2005; Smetana et al, 2001) (Fig. 4.6 A lower panel, green) in comparison with cells treated with only vehicle which showed normal distribution of NPM (Fig. 4.6 A upper panel, green). In cells treated with vehicle only, Filamin A was detected in the cytoskeleton, nucleus and nucleolus (Fig 4.6 A upper panel, red), whereas nucleolar localisation of Filamin A was completely lost in the presence of AMD (Fig 4.6 A lower panel, red). In control cells, 75% of cells presented the nucleolar Filamin A phenotype (FlnA-NOPhe), whereas cells treated with AMD showed a 100 % loss of FlnA-NoPhe (Fig. 4.6 B). These results show that the nucleolar retention of Filamin A is dependent on ongoing rDNA transcription.

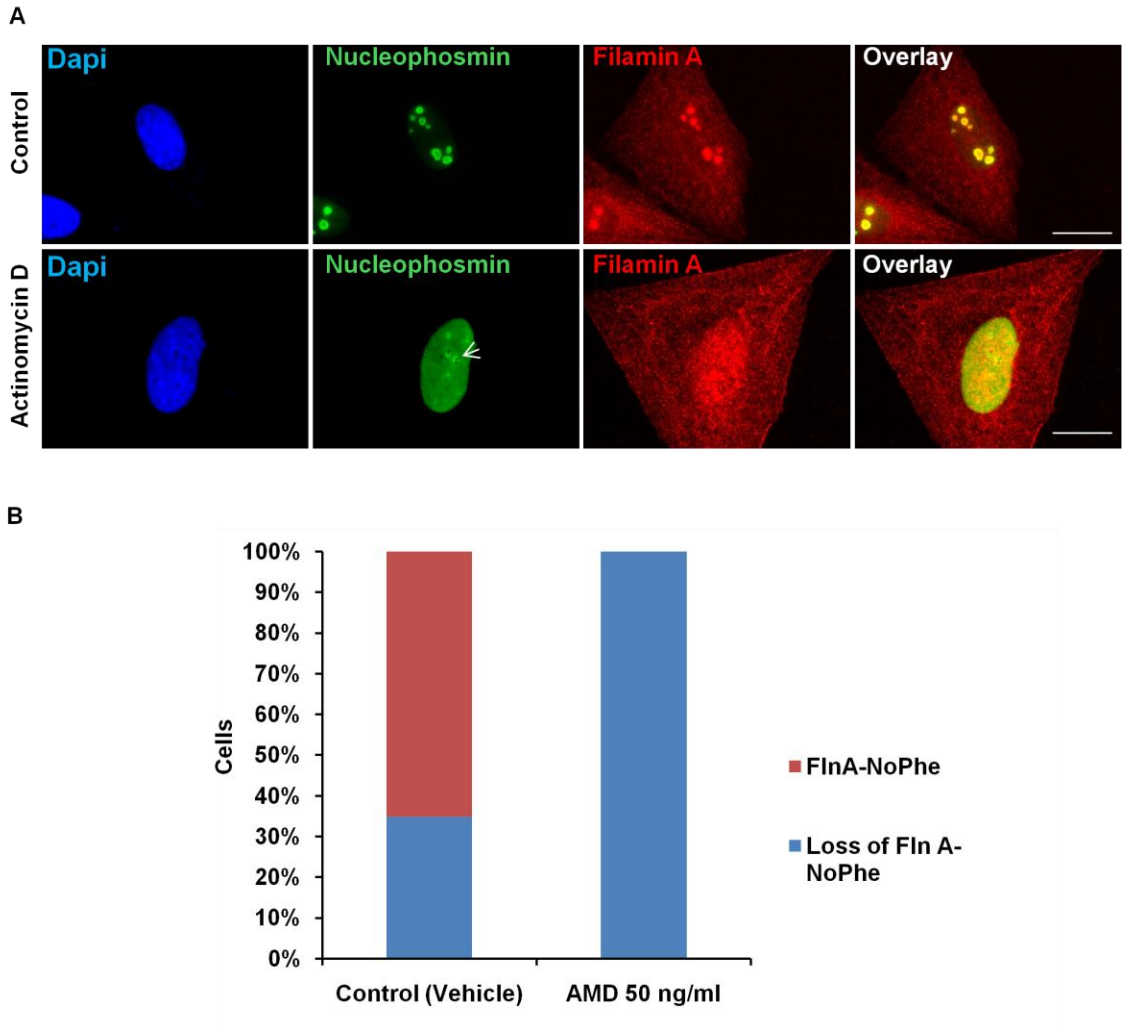


Fig. 4.6 Nucleolar retention of Filamin A depends on ongoing rRNA synthesis. (A) Filamin A (red) and NPM (green) immunofluorescence to show that blocking RNA polymerase I activity with actinomycin D (50 ng/ml) abrogates the nucleolar Filamin A phenotype (FlnA-NoPhe), whereas cells treated with only vehicle showed no changes in FlnA-NoPhe. White arrow show the typical cap-shape in the nucleolar periphery induced by AMD . Bar, 10 µm. (B) Percentage of cells showing FlnA-NoPhe and cells with loss of FlnA-NoPhe was estimated in both control and AMD treated cells. Nuclei (blue) were stained with DAPI.

4.5 Filamin A affects ribosomal RNA (rRNA) expression

The nucleolus is a subnuclear organelle formed around the ribosomal DNA (rDNA) repeats, which cluster at chromosomal loci called nucleolar organizing regions (NORs). In the nucleolus, the 28S, 18S and 5.8S ribosomal RNAs (rRNAs) are transcribed, processed and assembled into ribosomal subunits (McStay & Grummt, 2008; Moss et al, 2007). The ribosomal genes, except the 5S gene, are transcribed only by RNA polymerase I (Pol I), which is regulated by a set of nucleolar proteins (Sirri et al, 2008). Since Filamin A was found to be localised to the nucleolar compartment with the Pol I subunit RPA40 I sought to determine whether Filamin A affected rRNA expression. cDNAs from Filamin A-negative (M2) and Filamin A-positive (A7) cells was subjected to RT-qPCR using primers designed to amplify Filamin A, 28S and pre-RNA human ribosomal genes (Fig. 4.7). Ribosomal 28S expression markedly increased 3 fold in M2 cells in comparison with A7 cells. Also, more than 2 fold increase of pre-RNA levels was observed in the Filamin A-negative cell line M2 in comparison with the Filamin A-positive cell line A7. Thus rRNA expression was significantly increased in the absence of Filamin A.

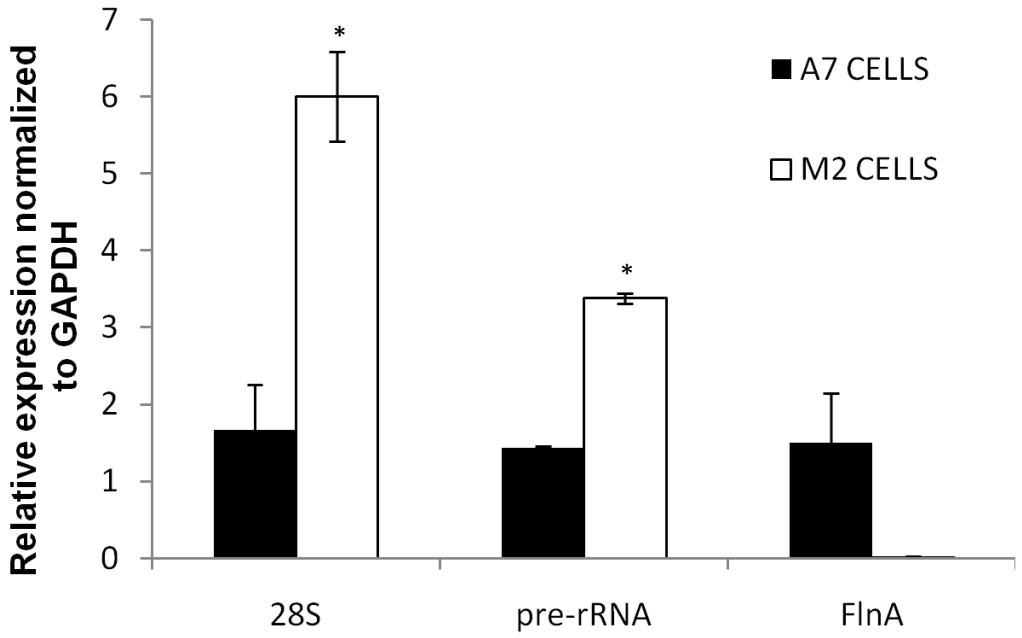
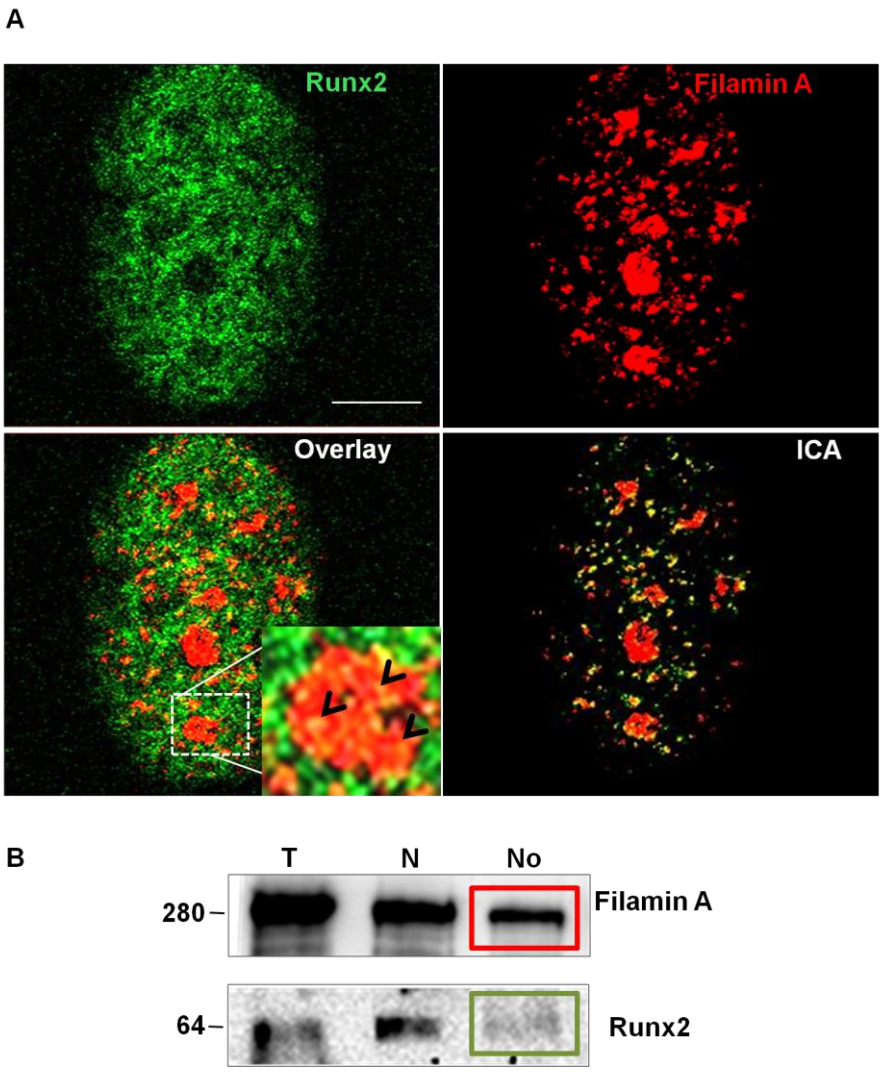


Fig. 4.7 Ribosomal gene expression in Filamin A negative versus Filamin A-positive cells. A7and M2 cells were grown at 80% confluence, harvested and RNA extracted for RT-qPCR purposes. Relative expression of the 28S and pre-RNA and Filamin A mRNA was determined by using the $2^{-\Delta\Delta ct}$ method. GAPDH was used as a reference housekeeping gene. Data are presented as mean ± standard deviation (S.D.) (n = 3). Statistical evaluation of significant differences was performed using the Student's t-test. Asterisk (*) indicates $P < 0.05$ when compared to control A7 cells.

4.6 Nucleolar Runx2 and Filamin A coexist in the nucleolus.

In this thesis, the interaction between Filamin A and Runx2 has been demonstrated. Runx2 is a transcription factor which regulates RNA polymerase II transcribed genes. However, Runx2 has also been found in the nucleolus to negatively regulate ribosomal gene expression driven by RNA polymerase I (Ali et al, 2010; Young et al, 2007). Filamin A and Runx2 interact and both proteins can be located in the nucleolus. Therefore, Filamin A might affect the regulation of rRNA expression via Runx2. First, the localisation of Filamin A and Runx2 in the nucleolus by confocal microscopy was determined in SaOS-2 cells. Runx2 staining showed the typical Runx2 distribution throughout the nucleus but also in the nucleolar compartment (Fig. 4.8 A, green panel). This result is in agreement with the findings of Young et al, 2007, where a small proportion of Runx2 was localised to the nucleolus. Filamin A staining is shown to be located in the nucleus and also abundantly expressed in the nucleolus (Fig. 4.8 A, red panel). When the two images (green and red) were merged a colocalisation signal was observed (Fig. 4.9 A, overlay panel, snapshot zoom). Colocalisation was later corroborated by performing an intensity correlation analysis (ICA) (Li et al, 2004). ICA is a statistical method which determines the synchrony of two proteins in a complex to show either total, random or anti colocalisation. By using ICA I demonstrated total colocalisation of Runx2 (yellow) with Filamin A (red) in both the nucleus and nucleolus (Fig. 4.8 A, lower panel, right). Nucleolar localisation of Runx2 and Filamin A were also confirmed by western blot (Fig. 4.8 B). These data demonstrate that Filamin A and Runx2 interact within the nucleolus.



4.8 Filamin A and Runx2 colocalise in the nucleolar compartment. SaOS-2 cells were fixed and processed for immunostaing against Filamin A and Runx2. (A) Confocal analysis shows colocalisation of those proteins in the nucleolar compartment as analyzed by Intensity correlation analysis (Lin et al, 2006). Bar, 5 μm. (B) Western blot analysis of Total (T) Nuclear (N) and Nucleolar (No) fractions tested against Filamin A and Runx2 to show those proteins coexist in the nucleolus.

4.7 Runx2 and Filamin A synergistically repress ribosomal DNA expression.

Given that both Filamin A and Runx2 repress rRNA expression and that they interact within the nucleolus I next determined whether Filamin A inhibits rRNA expression via Runx2. To do this, the activity of a mouse rDNA promoter was measured in Filamin A-knockdown MC3T3 cells. The mouse wild-type rDNA promoter (rDNApWT) contains a proximal Runx site in its sequence (Budde & Grummt, 1999) (Fig. 4.9 A). rDNApMut was generated by mutating the Runx binding-site from TGAGGT to TGAACT. Filamin A expression was knocked down in MC3T3 as described in chapter 3. Four days post-transfection cells were transfected again with either rDNApWT or rDNApMut. mRNA from cells was isolated, cDNA prepared and RT-qPCR was performed to amplify a specific sequence contained in the reporter gene. Co-transfection of Filamin A siRNA with rDNApWT resulted in 1.7 fold increase in the promoter activity compared to control siRNA transfections (Fig. 4.9 C, black bars). This result correlates with the increase in rRNA expression observed in Filamin A-negative M2 cells (Fig. 4.7). Importantly, when the Runx2-binding sites in the rDNApWT were mutated a significant increase of the activity of the promoter was observed in both Filamin A siRNA and control siRNA transfected cells (Fig. 4.9 C, white bars). These data demonstrate that knockdown of Filamin A results in an increase in the activity of the rDNA promoter, suggesting that Filamin A represses transcription from this promoter. However, this regulation does not prove that Filamin A is regulating directly ribosomal gene transcription. In order to show that Filamin A had a direct effect on the regulation of rRNA, Filamin A was introduced in the Filamin A-negative M2 cell line (Fig. 4.9 D). M2 cells were transfected with either two versions of the full length Filamin A (GFP-

FlnA and Myc-FlnA) or plasmid control. Later, the ribosomal DNA reporter plasmid (rDNAp) was transfected. After 24 h the RNA was isolated, cDNA prepared and RT-qPCR was performed. The primers used to detect the rDNAp expression were: ATT CAC TGG CCG TCG TTT TA (Fwd) and GGC CTC TTC GCT ATT ACG C (Rev). As expected, M2 cells transfected with a plasmid control showed high levels of rDNA reporter activity (Fig. 4.0 D). In contrast, the reintroduction of GFP-Filamin A or Myc-Filamin A in M2 cells induced a drastic decrease on the activity of the rDNAp in comparison with M2 cells that received only the empty vector (Fig. 4.9 D). These findings suggest that exogenous expression of Filamin A in the Filamin A-negative M2 cell line suppresses the activity of the rDNAp. It has previously been demonstrated that Filamin A is downregulated in differentiating MC3T3 cells (Chapter 3). As it has been suggested that Filamin A might suppress the activity of the ribosomal DNA promoter; I sought to determine whether rDNA promoter activity increases in these cells during differentiation. To do this, the activity of the rDNApWT was measured into non-differentiating and differentiating MC3T3 cells. The downregulation of Filamin A in differentiated cells is shown (Fig 4.10 A) in comparison to control cells. At day eight after induction of differentiation, differentiated and control MC3T3 cells were transfected with rDNApWT and the promoter activity determined 48 h later (Fig. 4.10 B). The activity of the promoter increased 3 fold in differentiating cells in which Filamin A is downregulated in comparison with undifferentiated cells. Thus a reduction in Filamin A expression, induced by siRNA or during differentiation, correlates with an increase in the activity of the rDNA promoter. These findings suggest that Filamin A suppresses the activity of the rDNA promoter.

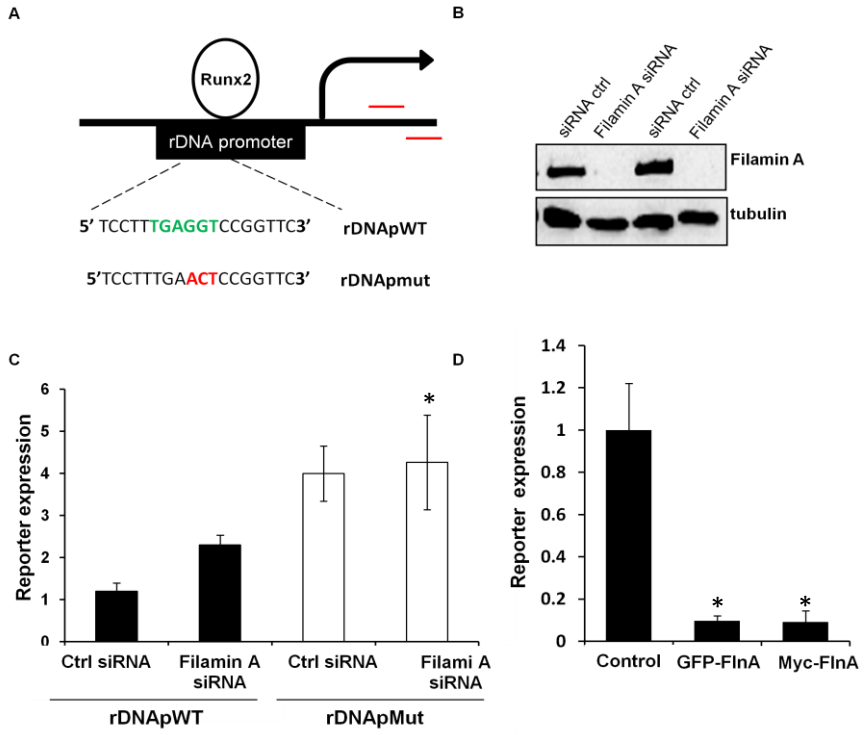


Fig.4.9 Filamin A and Runx2 act as repressors of ribosomal DNA (rDNA) expression. (A) Schematic representation of the rDNA wild type promoter reporter plasmid showing the Runx2 sites in green. The induced Runx2 site mutations in order to produce rDNApMut are shown in red. (B) Western blot from MC3T3 cells transfected with either control or Filamin A siRNA to show the downregulation of Filamin A. Tubulin was used as a loading control. (C) siRNA knockdown cells were transfected with either rDNApWT or rDNApMut as indicated and RT-qPCR based reporter assay was performed. Data are presented as mean ± standard deviation (S.D.) (n = 3). Statistical evaluation of significant differences was performed using the Student's t-test. Asterisk (*) indicates $P < 0.05$ when compared to control siRNA cells transfected with rDNApWT. (D) RT-qPCR analysis shows that the activity of the rDNAp is reduced after transfection of two different versions of Filamin A in the Filamin A-negative M2 cell line. * indicates $P < 0.05$ when compared to control empty vector cells.

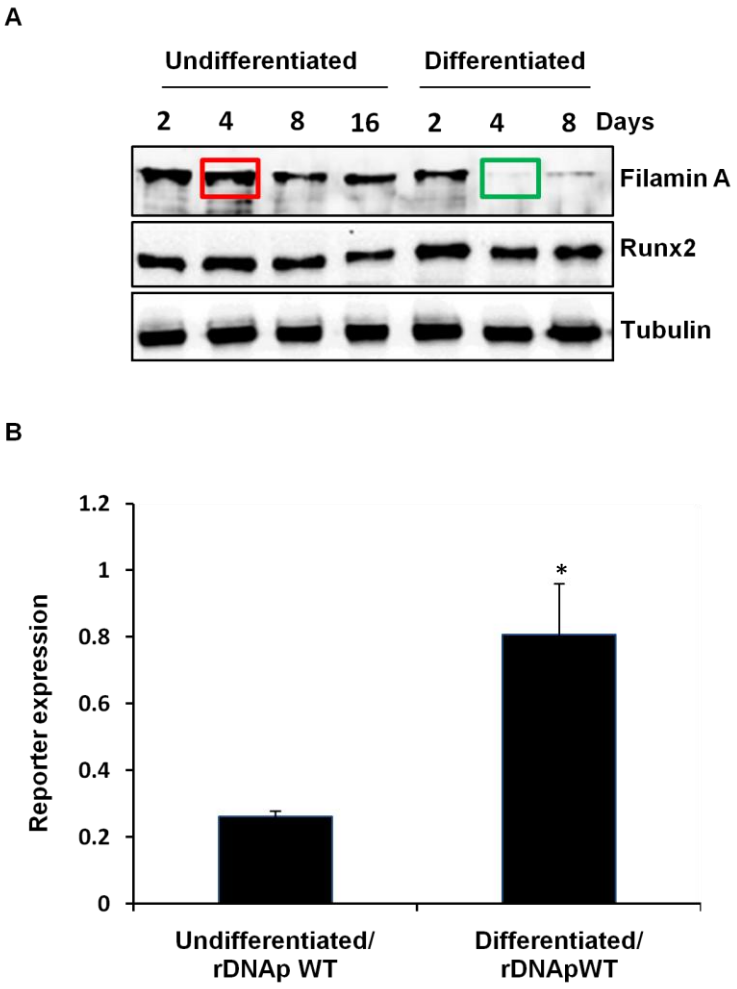


Fig. 4.10 Differentiating MC3T3 cells display an increase of ribosomal expression. (A) Western blot from undifferentiated and differentiated MC3T3 cells to show the natural downregulation of Filamin A. Molecular mass is presented on the left and tubulin was used as a loading control. (B) Undifferentiated and differentiated MC3T3 cells from day four were transfected with rDNApWT as indicated and RT-qPCR based reporter assay was performed. Data are presented as mean ± standard deviation (S.D.) (n = 3). Statistical evaluation of significant differences was performed using the Student's t-test. Asterisk (*) indicates $P < 0.05$ when compared to control cells (Undifferentiated/rDNApWT).

Having demonstrated that Filamin A and Runx2 interact in the nucleolus and that Filamin A can suppress ribosomal gene expression it was next sought to determine if Filamin A was recruited to the promoter of the 18S and 28S ribosomal genes by ChIP assays in SaOS-2 cells. This experiment was performed by Dr WengSheng Deng, a current postdoc in our group. RNA polymerase I transcribe nuclear genes for ribosomal RNA, thus important for ribosomal biogenesis. The Pol I subunit, RPA40, is an essential subunit that is associated in early steps of RNA Pol I assembly, giving integrity to the complex (Song et al, 1994). Therefore, Filamin A and RPA40 antibodies were used to pull-down protein-DNA complexes and IgG was used as a control. Endogenous Filamin A and RPA40 were both present on the endogenous 18S and 28S promoters in SaOS-2 cells when compared to control IgG experiments (Fig. 4.11). Taken together the data presented in this chapter demonstrates that Filamin A interacts with Runx2 in the nucleolus to directly suppress ribosomal DNA expression.

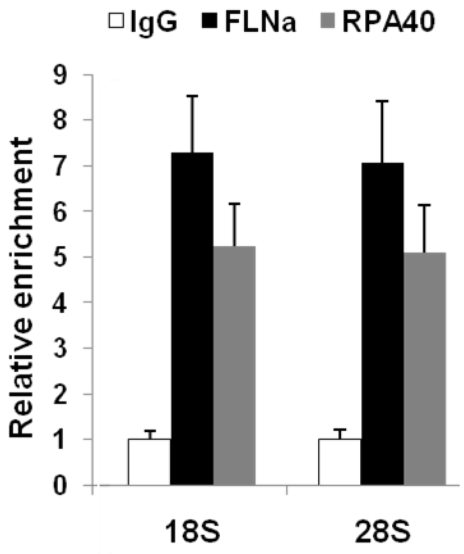


Fig. 4.11 Chromatin immunoprecipitation (ChIP) analysis shows that Filamin A is associated to the rDNA promoter. ChIP assays showing Filamin A and RPA40 are associated to the rDNA promoter. ChIP assays were performed with antibodies indicated on the top of the graph. The data show RT-qPCR performed with 18S and 28S. Experiment performed by Dr. WengSheng Deng.

4.9 Discussion

The data presented in this study demonstrates for the first time that Filamin A is present in the nucleolus. Also it has been shown that Filamin A form a complex with Runx2 to negatively regulate the activity of ribosomal genes by binding to the rDNA promoter.

4.9.1 Filamin A is found in the nucleolus.

In this study, it has been shown by immunofluorescence and western blot analysis that a considerable amount of endogenous Filamin A localises to the nucleolus in osteoblastic cell lines as well as in primary mouse cells. Also, the nucleolar localisation of Filamin A in HeLa cells and in the stably Filamin A-positive (A7), in comparison with the Filamin A-negative (M2) melanoma cell line was confirmed. The high purification of nucleoli performed in this project is identical to the one used to elucidate the Nucleolar Proteome, conducted mainly by Professor Angus Lamond (Ahmad et al, 2009). By using high sensitivity mass spectrometry and stringent criteria, Lamond's group has so far identified more than 50,000 peptides corresponding to over 4,500 human proteins from purified nucleoli (Ahmad et al, 2009). Given the experimental evidence in this thesis which shows Filamin A in the nucleolus, I search if Filamin A could be a member of the list in the Nucleolar Proteome Database (NOPdb) published recently (www.lamondlab.com/NOPdb). Search for Filamin A in the NOPdb showed that Filamin A is listed as member of the nucleolar proteome list (Fig. 4.12). A total of 103 peptides were identified by mass spectrometry analysis. Alignment of those peptides covered in a wide extent of the full length sequence of Filamin A (not shown). In this thesis Filamin A has been identified, and corroborated with the data acquired from the nucleolar proteome database, as a new member to the list of nucleolar proteins.

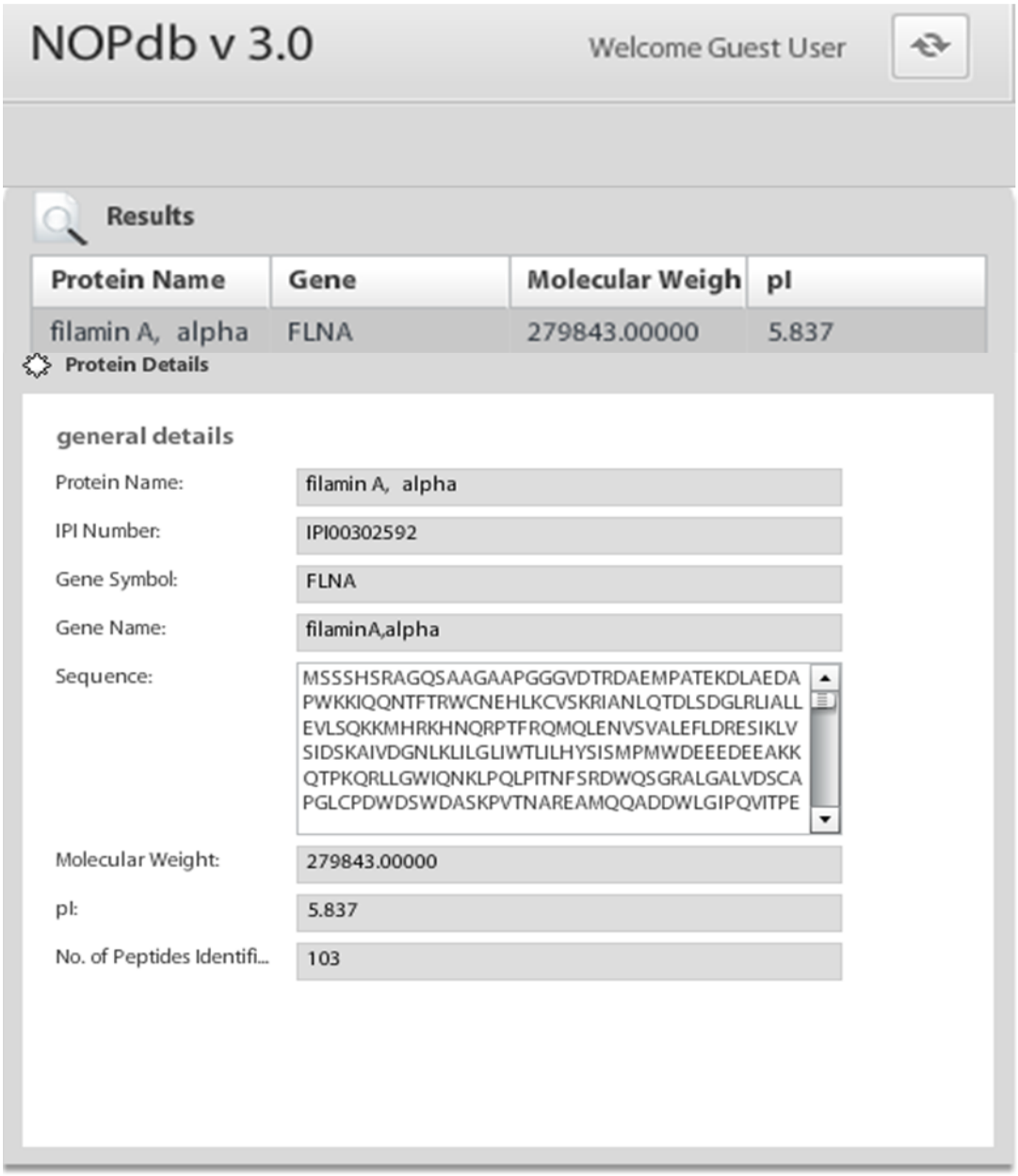


Fig. 4.12 Filamin A is present in the Nucleolar Proteome Database. A search in the nucleolar database internet (http://www.lamondlab.com/NOPdb3.0/) shows Filamin A is present in the purified nucleoli extract from HeLa cells as identified by mass spectrometry. The search displays the name, symbol, sequence, molecular weight, isoelectric point (pI) and number of peptides identified by mass spectrometry.

4.9.1.1 A candidate nucleolar localisation signal in Filamin A

Nucleolar Localisation Signals (NoLSs) are often characterised by a consecutive set of basic amino acids (Emmott & Hiscox, 2009). Given the observations that indicate Filamin A can be located in the nucleolus, a search for NoLS in the Filamin A protein sequence was made. Filamin A contains a conserved RRXR motif located in the position 2138 amino acid sequence (Fig. 4.13 A), which is close to the carboxy-terminal end. This motif has been also found in proven nucleolar associated proteins like Parp2, p14/19Arf, ING1b, Rpp29 or HIV Tat (Jarrous et al, 1999; Kubota et al, 1989; Lindstrom et al, 2000; Meder et al, 2005; Scott et al, 2001) (Fig. 4.13 B). As a preliminary experiment I examined whether the carboxy terminal domain of Filamin A, which contains a candidate NoLS, is able to translocate into the nucleolus. A 100 kDa carboxy terminal fragment of Filamin A (Fig. 4.13, C) tagged with GFP (pEGFP-C2 FlnA 16-24) was transfected into HeLa cells along with the control vector pEGFP-C2. and its intracellular localisation was analysed. Fig. 4.13, D shows that the carboxy terminus domain is efficient to retain Filamin A-GFP in the nucleolus in almost 50% of transfected cells after 72 h of transfection. This result correlated with nucleolar staining of endogenous Filamin A that could not be observed in all the cells. This observation could be due to a specific cellular activity. Also it would be important to determine the nucleolar localisation of a Filamin A-GFP stable cell line in long term cultures. In this regard it has been published that in long-term cultures of cells that stably express the wild type extended-C/EBPa isoform, the percentage of cells with nucleolar localisation of extended-C/EBPa continuously increases in a long term period (Muller et al, 2010).

The authors discussed that it may be a mechanism that stimulates nucleolar retention; this might also apply to Filamin A nucleolar retention.

In terms of nucleolar retention, it has been reported that the RRXR motif is required for the interaction with the nucleolar protein NPM (Meder et al, 2005; Muller et al, 2010) which has been shown in this thesis to colocalise with Filamin A. This could mean that either Filamin A needs to be nucleolar to interact with nucleophosmin or that Filamin A is localised to the nucleolus through binding to nucleophosmin. Hence, these results suggest that the possible nucleolar localisation could be an important feature of Filamin A and the RRXR NoLS-motif contained in the carboxy terminus domain might be required for its nucleolar retention. To effectively deal with those possibilities more studies are required to determine the mechanism in which Filamin A can travel and resides, either temporarily or permanently in the nucleolus.

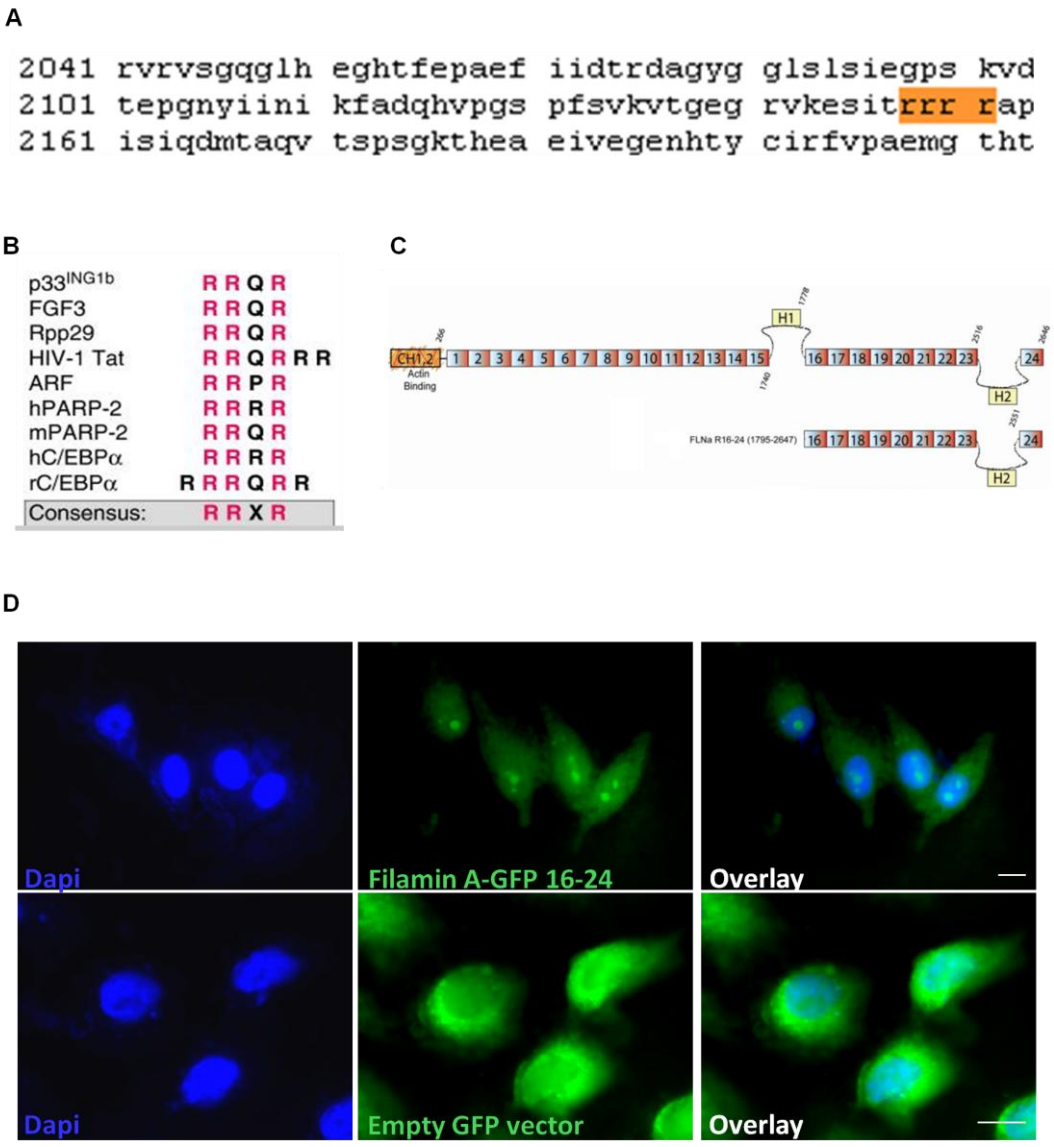


Fig. 4.13 Filamin A contains a nucleolar localisation signal (NoLS). (A) A portion of the Filamin A sequences where the possible RRXR motif is found. (B) Alignment of the RRXR motifs within the domains mediating nucleolar localisation of proteins with known nucleolar localisation. (C) Schematic representation of Filamin A fragments transfected into HeLa cells. Top and bottom schemes represent full length and 100 kDa Filamin A (D) Immunofluorescence of cells transfected with 100 kDa Filamin A as indicated. Nuclei (blue) were stained with DAPI. Bars, 10 μm.

4.9.2 Nucleolar Filamin A and Runx2 regulate ribosomal DNA expression

The nucleolus is the site of ribosome biogenesis. A wide amount of reports have shown that the nucleolus has other functions such as regulations of cell-cycle and cellular stress response (Carmo-Fonseca et al, 2000; Olson et al, 2000; Pederson, 1998). Filamin A accumulates in the nucleolus, indicating that it may have a nucleolar function. The internal structure of the nucleolus has been described in detail by microscopy and three well defined compartments have been identified: the fibrillar centres (FCs), which are surrounded by dense fibrillar components (DFCs), and the FC-DFC complexes are surrounded by the granular component (GC) (Junera et al, 1995; Vandelaer et al, 1996). Each compartment has been shown to specifically harbour a considerable amount of proteins. Some of them are ubiquitous residents of each subcompartment which suggested that each compartment can perform different functions. However, some proteins can accumulate in one or two of these subcompartments at the same time (Schwarzacher & Mosgoeller, 2000). In one effort to determine the subnucleolar localisation of Filamin A, a set of different antibodies were used to co-label the nucleolus with Filamin A. In this study it is suggested that in interphase cells Filamin A is located in the granular compartment and in the fibrillar centre, as colocalisation with nucleophosmin and the Pol I subunit RPA40 could be detected. These compartments are well known to be involved in the regulation of ribosomal DNA transcription.

In this work it has been shown that the rDNA promoter can be synergistically regulated by Filamin A and Runx2 by studying the activity of the rDNA promoter which had its Runx2 site mutated. Since Filamin A and Runx2 have been shown to interact, it is suggested that this interaction might happen as well in the nucleolus. It has been

reported that Runx2 associates directly with ribosomal DNA during interphase and mitosis and interacts with SL1 complex and the upstream binding factor (UBF) that is required for the pre-ribosomal RNA transcription (Young et al, 2007). Therefore, the results obtained in this work corroborate the important function of Runx2 in regulating the rDNA expression but also have shown that Filamin A and Runx2 complex could impact the activity of the ribosomal genes. It is well documented that actin, another cytoskeletal protein which Filamin A binds to, can impact as well the rRNA transcription (Castano et al, 2010; Obrdlik et al, 2007; Skarp & Vartiainen, 2010; Visa & Percipalle, 2010). Therefore, it is possible that cytoskeletal proteins can regulate rDNA transcription driven by RNA polymerase I. In this regard, Filamin A is involved in this process since nucleolar retention of Filamin A depends on ongoing rDNA transcription, as actinomycin D experiments have suggested in this thesis. The initial findings presented in this thesis suggest that Filamin A could have a potential role in nucleolar RNA processing. Also, it has been shown that the absence of Filamin A favours rDNA transcription, suggesting that Filamin A is involved in the regulation of ribosomal DNA expression by RNA polymerase I. These findings are supported by ChIP assays experiments made in Paul Shore's group by Dr. Wengsheng Deng which have revealed the association of Filamin A with rDNA promoter sequences.

4.9.3 A suggested mechanism of the regulation of ribosomal genes by Filamin A.

Given that Filamin A and Runx2 has been shown in this work to be located in the nucleolus to regulate negatively the rRNA expression a model of Filamin A function is suggested (Fig. 4.14). In this model it is proposed that Filamin A and Runx2 are present but not bound to the rDNA promoter, therefore the ribosomal transcription is active. After one stimulus, such rDNA transcription is silenced as Runx2 and Filamin A are associated to the promoter. This can be the case for cells that are under the mitotic process. In this process the cell stops the production of ribosomal DNA and decrease the activity of translation of proteins by ribosomes. However, as not all the cells expressed Runx2, it could be thought that this repressional mechanism can be the same case for cells that also express both Runx1 and Runx3 proteins. The mechanism of how Filamin A and Runx2 suppress the activity of ribosomal genes currently is not known; however it is clear that Filamin A could be an important player in maintaining the levels of rRNA transcription and therefore it could regulate the rate of protein synthesis, the cellular activity, cell proliferation and cell size.

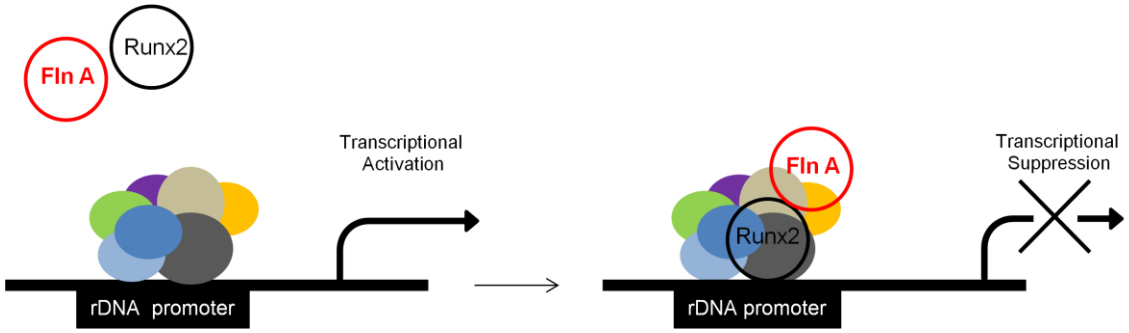


Fig. 4.14 A model depicting control of rDNA transcription by Filamin A and Runx2. Filamin A and Runx2 are present in the nucleoli but not located in the promoter of the rDNA, so the rDNA is transcriptionaly active. In certain circumstances, Filamin A and Runx2 are associated to the promoter, thus inhibiting rDNA transcription. This mechanism could be the case when cells are preparing to entering mitosis.

5.0. Investigating new roles of the Filamin A by gene microarray approach

5.1 Introduction

Genetic evidence links Filamin A as an essential protein for human development as Filamin A mutations have shown to produce bone malformation defects (Feng & Walsh, 2004). Moreover, reports have been published regarding the interaction between Filamin A and transcription factors (Popowicz, 2006) suggesting that Filamin A/transcription factor interaction might regulate gene expression. With this regard, it has been shown that CBFβ, the co-activator of Runx proteins associates with Filamin A. In this case Filamin A retains CBFβ in the cytoplasm, thus preventing CBFβ from entering the nucleus to bind Runx1 (Yoshida, 2005). In the previous two chapters it was demonstrated that Filamin A/Runx2 complex is present in the nucleus and nucleolus where it suppresses expression of MMP-13 and rRNA genes. To determine whether Filamin A regulates the expression of additional genes a microarray analysis was performed in the presence and absence of Filamin A.

Functional genomics involves the analysis of large amount of data derived from biological samples. One such type of large-scale experiment involves screening the expression levels of thousands of genes at the same time. This is called expression analysis or microarray. A microarray (Affymetrix GeneChip®) is a glass slide on to which DNA molecules are attached in specific spots. A microarray may contain thousands of spots and each spot contains DNA oligonucleotides that correspond to a unique gene. Affymetrix GeneChip® may be used to compare expression of a set of genes from a cell maintained in a particular condition to a control cell. Briefly, mRNA is extracted from the cells and the samples are flourescently labelled and allowed to hybridize onto the array. Any cRNA sequence in the sample will hybridise to specific

spots on the glass slide containing its complementary sequence. Following the hybridisation step, the fluorescent signal for each spot is scanned and acquired to be interpreted as an up or downregulated gene (Hacia et al, 1998). Here, microarrays were used to detect differences in gene expression between the Filamin A-negative cell line (M2) and their clonal derivatives which express Filamin A (A7). Interestingly some of the changes in expression were in bone-related genes. Given that M2 and A7 cells are melanoma cell lines, it was decided to extend the microarray analysis to the osteoblastic SaOS-2 cell line, in which Filamin A was depleted using siRNA. The results of this chapter demonstrate that there are significant differences in the gene expression induced by Filamin A ablation. Moreover, a subset of the differentially expressed genes is known to be involved in bone biology. The role of Filamin A as an important regulator of bone gene expression is discussed.

5.2 Microarray analysis in the Filamin A negative M2 cells

Microarray expression profiling was used to identify differentially regulated genes in the absence of Filamin A. Given the importance of Filamin A in cell signalling and gene regulation between others, it was aimed to explore to which extent Filamin A could impact, either directly or not, gene expression. By doing this, further evidence to support the idea of Filamin A as a regulator of gene expression could be provided.

The melanoma cell lines M2 and A7 are a convenient model to study Filamin A function. Therefore, the differences in gene expression between those cell lines were analysed. Briefly, M2 and A7 at 80% confluency were lysed and passed through a RNeasy column in order to purify the mRNA. The concentration of the RNA was

measured and the quality and control of the RNAs was assesed. Right after cDNA was prepared and labelled; the cRNA was loaded into the microarray (Affymetrix GeneChip® Human genome U133 array). This array provides a comprehensive coverage on the transcribed human genome and analyses the expression level of over 47,000 transcripts, including 38,500 well-characterised human genes. Expression transcripts were quantified in the Filamin A-negative M2 cell versus the control, the Filamin A-positive, A7 cell line. The microarray experiment was performed in triplicate. The upregulation of 1546 genes and the downregulation of 1404 genes in M2 cells when compared to A7 cells was detected (Fig. 5.0 A).

The list of differentially expressed genes was subsequently submitted to the Database for annotation, visualization and integrated discovery (DAVID). This is a database that provides gene clustering and functional data of a given list of genes (Huang da et al, 2009). DAVID was set to cluster groups of genes involved in bone-related biology. In M2 cells, a total of 71 bone related genes were differentially regulated; 35 were upregulated and 36 genes were downregulated (Fig. 5.0 B). Differentially regulated bone-related genes in M2 cells are shown as a heat map diagram (Fig. 5.1). Microarray maps are a special type of heat map used to highlight gene expression. Each row of the heat map represents a gene, and each column represents an independent biological sample (in this case, triplicates). In this study, the genes shown in the microarray heat map are important for promoting and maintaining bone homeostasis and mineralization (Fig. 5.1). These data suggest that Filamin A expression affects the expression of a number of bone-related genes in melanoma cells.

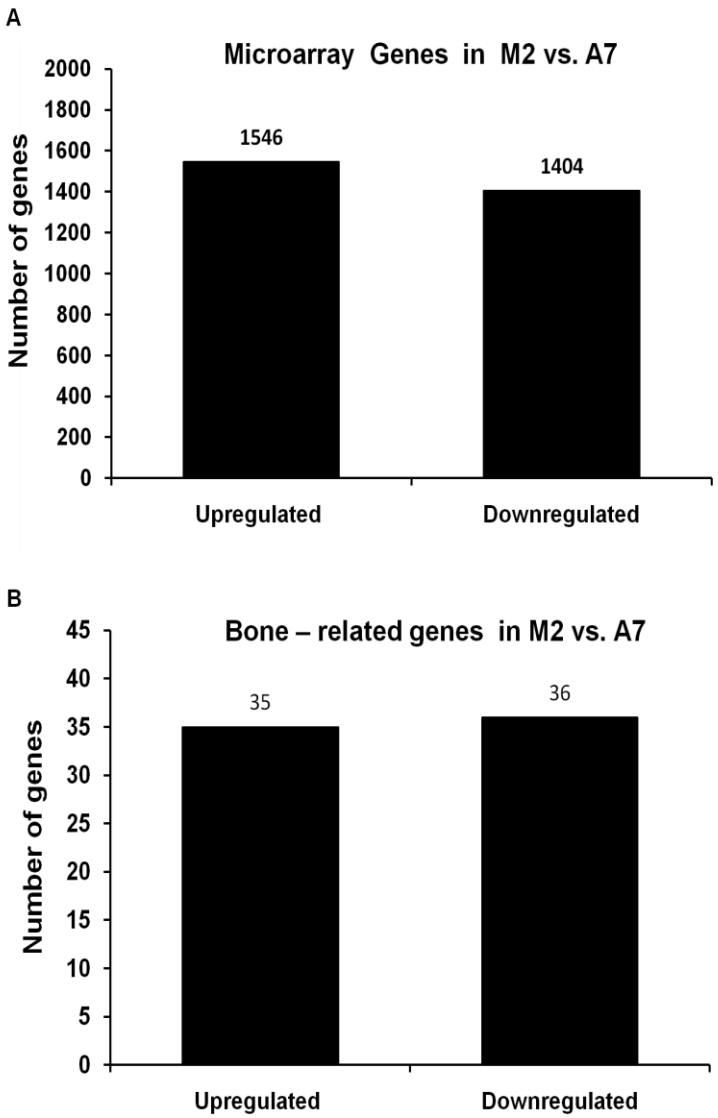


Fig. 5.0 Filamin A dependent gene expression in M2 cells. (A) Microarray analysis was performed in the Filamin A-negative M2 cell line versus its control the Filamin A-positive A7 cell line. The graph shows the total amount of upregulated or downregulated genes when Filamin A is absent. (B) Differential regulation of bone-related genes in M2 cells**.** DAVID analysis was performed to cluster genes related to skeletal biology. The graph shows the total amount of upregulated or downregulated bone-related genes when Filamin A is absent.

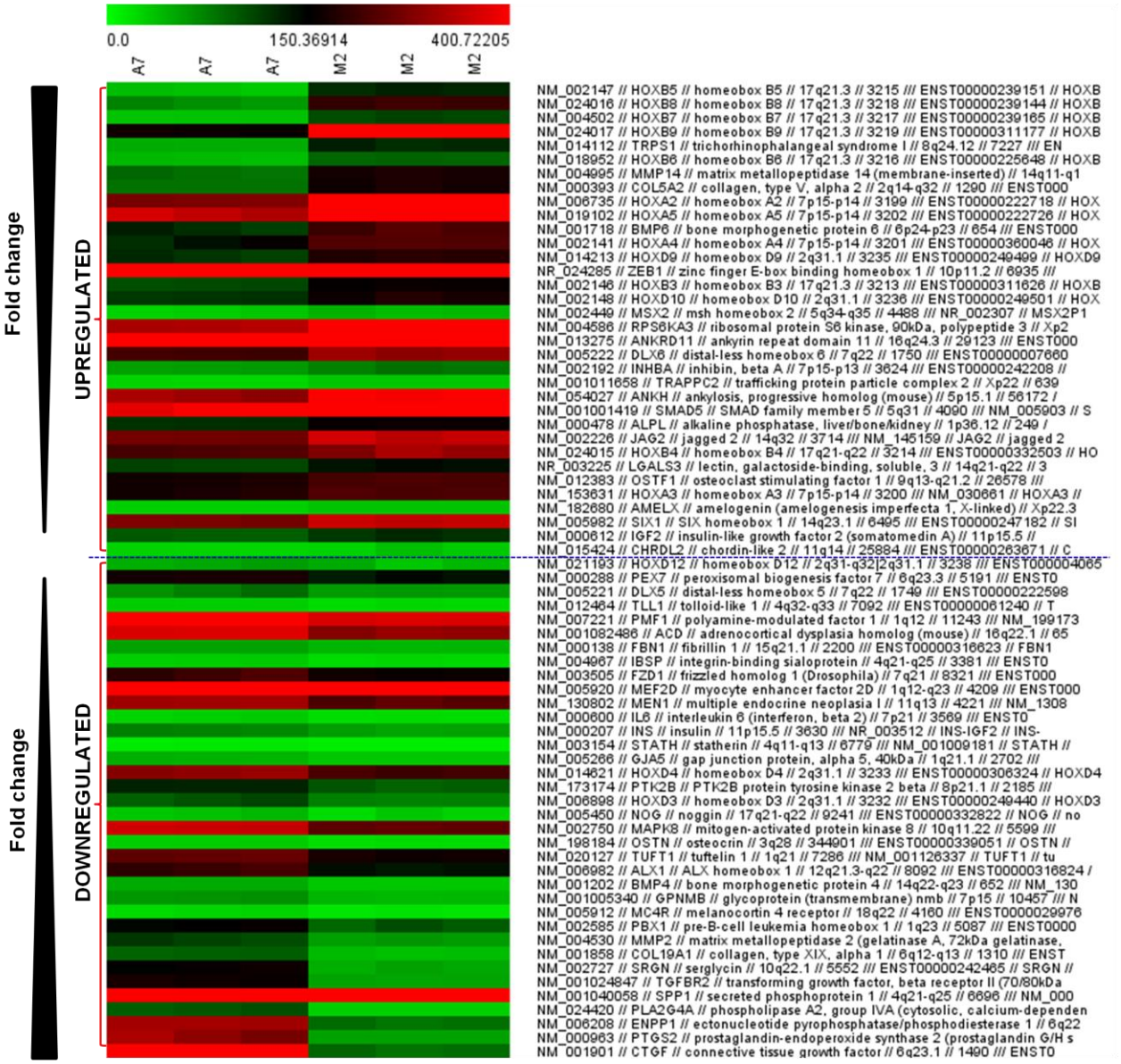


Fig. 5.1 Heat map diagram showing the expression profiling of bone-related genes in M2 and A7 cells. Red indicates transcriptional activation and green represents repression. Black indicates fold change expression is not markedly altered. Transcripts that are upregulated or downregulated are shown above or below the blue dotted line, respectively. The list is organised according to the highest fold change between the genes analysed (left black arrows).

5.3 Microarray analysis of Filamin A negative osteoblastic cells

It has been established that Filamin A affects gene expression and a group of bone-related genes in M2 versus A7. However, it is important to mention that these cells are melanoma cell lines and that they do not express Runx2. Nonetheles, the differential expression observed in those cell lines provides evidence on the regulation of gene expression by Filamin A. Therefore, the gene expression changes in SaOS-2 cells in the presence and absence of Filamin A using siRNA was analysed. As SaOS-2 cells are an osteoblastic cell line and in consequence express Runx2, changes in gene expression induced by Filamin A downregulation could provide a better understanding of how Filamin A affects bone formation. In SaOS-2 cells, transfection with Filamin A-siRNA reduced the amount of Filamin A by 90% (Fig. 5.2 A). Microarray analysis of the mRNA from these cells revealed that a total of 979 genes were significantly upregulated and 936 genes were significantly downregulated (Fig. 5.2 B). DAVID annotation gene cluster analysis for genes related to bone biology was performed from the microarray analysis in the Filamin A-siRNA depleted SaOS-2 cell line versus wild type SaOS-2. The results show that in Filamin A-knockdown SaOS-2 cells, a total of 41 bone-related genes were differentially regulated; 23 genes were upregulated and 18 genes were downregulated (Fig. 5.2 C). Differentially regulated bone-related genes in Filamin A-knockdown SaOS-2 cells are shown as a heat map diagram (Fig. 5.3).

In summary, by microarray analysis, it was found that in the osteoblastic cell line SaOS-2 many genes related to bone biology are affected by depleting Filamin A. This data, along with the M2 microarray data demonstrates that Filamin A may have a role in the regulation of bone-gene expression.

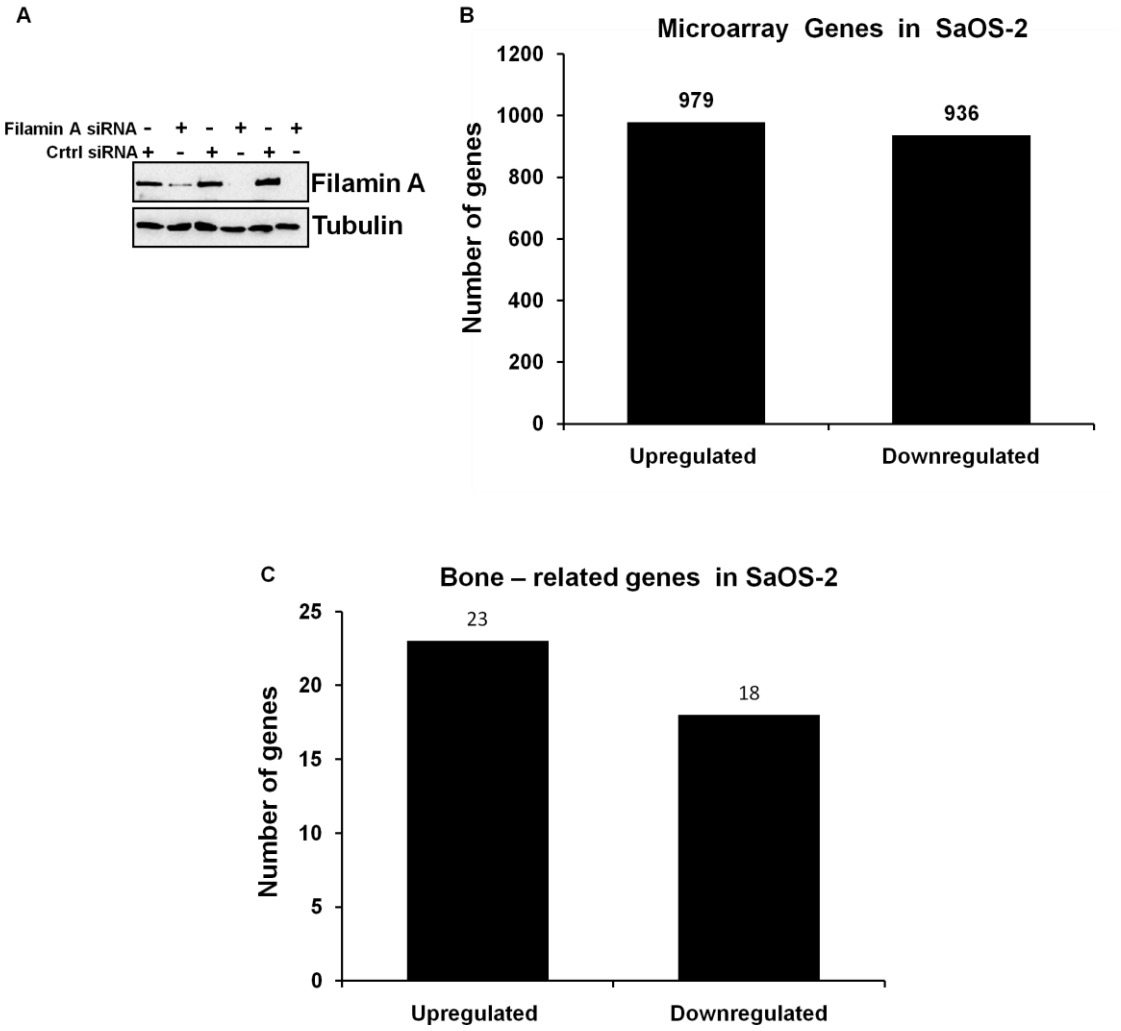


Fig. 5.2 Filamin A dependent gene expression in SaOS-2 cells. Microarray analysis was performed in the Filamin A-knockdown osteosarcoma cell line SaOS-2 versus its control, SaOS-2 transfected with scrambled siRNA. (A) Expression of Filamin A was analyzed by Western blot in cell lysates from SaOS-2 cells transfected with siRNA targeting Filamin A and siRNA control; similar results were obtained in three independent experiments. (B) The graph shows the amount of upregulated or downregulated genes when Filamin was depleted. (C) Differential regulation of bone related genes in SaOS-2 cells**.** DAVID analysis was performed to cluster genes related to skeletal biology.

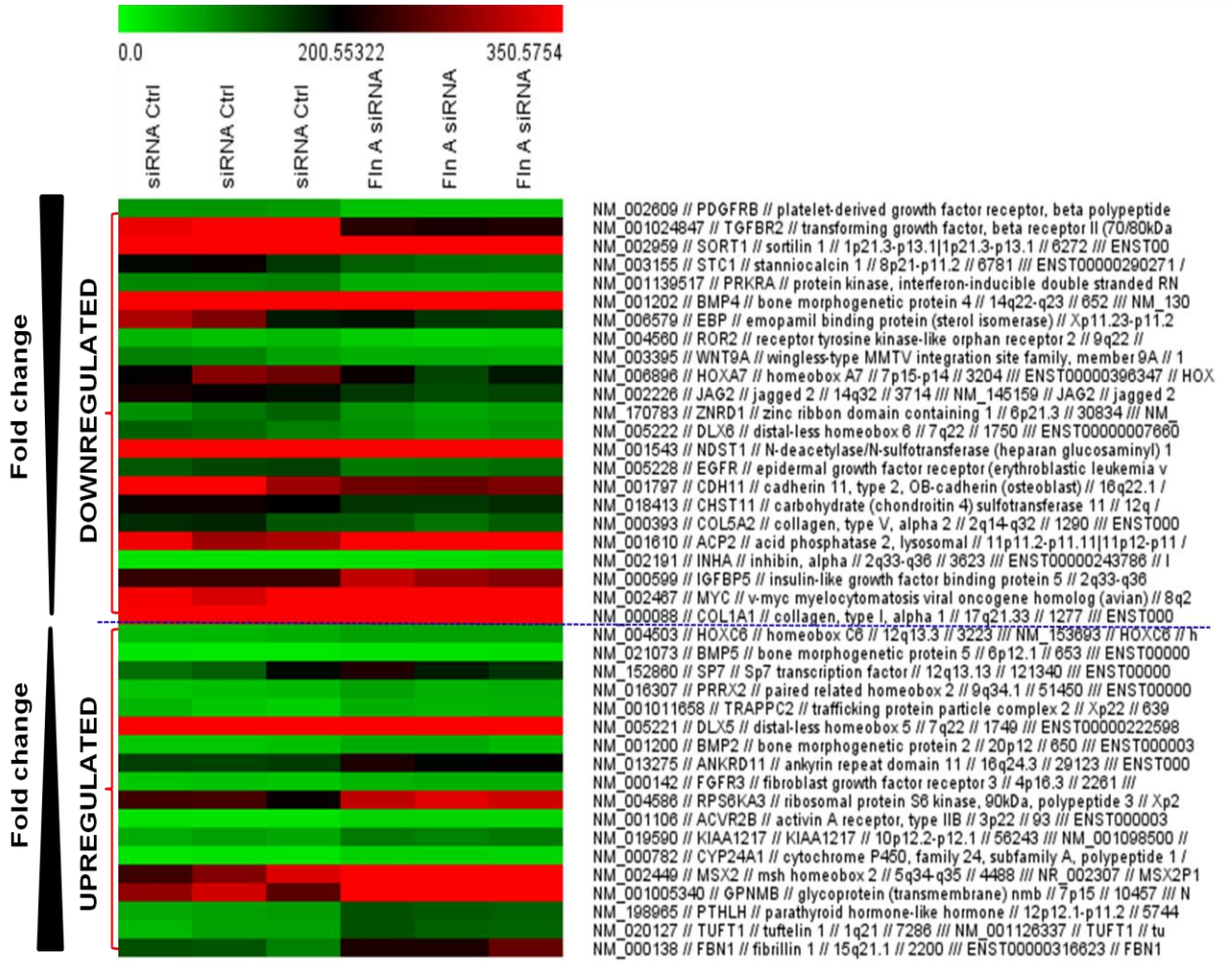


Fig. 5.3 Heat map diagram showing the expression profiling of the differentially regulated bone related transcripts when Filamin A is downregulated in SaOS-2 cells. Red indicates transcriptional activation and green represents repression. Black indicates fold change expression is not markedly altered. Transcripts that are upregulated or downregulated are shown above or below the blue dotted line, respectively. The list is organised according to the highest fold change between the genes analysed (left black arrows).

5.4 Comparison between M2 and SaOS-2 microarray data in terms of bone-related genes

It has been suggested that bone related genes are affected by the absence of Filamin A in M2 and SaOS-2. Therefore, a Venn diagram was built aimed to identify which genes, derived from the microarray analysis, were common on both cell lines. As it can be seen in Fig. 5.4, from the 71 differentially regulated genes in M2 cells (red circle), 12 genes were overlapped (yellow zone) when compared to the 41 differentially expressed genes shown in SaOS-2 cells (green circle) (Fig. 5.4). The bone related genes that were common in both microarrays are: msh homeobox 2, ribosomal protein S6 kinase, trafficking protein particle complex 2, TGF- beta receptor II, BMP-4 collagen type V, alpha 2, fibrillin 1, tuftelin, glycoprotein NMB, jagged 2, distal-less homeobox 6, distal-less homeobox 5. Also, a list of bone related representative genes that did not overlapped was made. In M2 cells the selected genes were: MMP-14, BMP-6, alkaline phosphatase, osteoclast stimulating factor 1, Conective Tissue Growth factor (CTGF), MMP-2, Osteocrin and Noggin. In SaOS-2 cells the selected genes were: parathyroid hormone-like hormone, BMP-2, Osterix, BMP-5, collagen, type 1, alpha 1 and inhibin alpha. The selected genes along with the ID gene, abbreviation and function are shown in table 5.0.

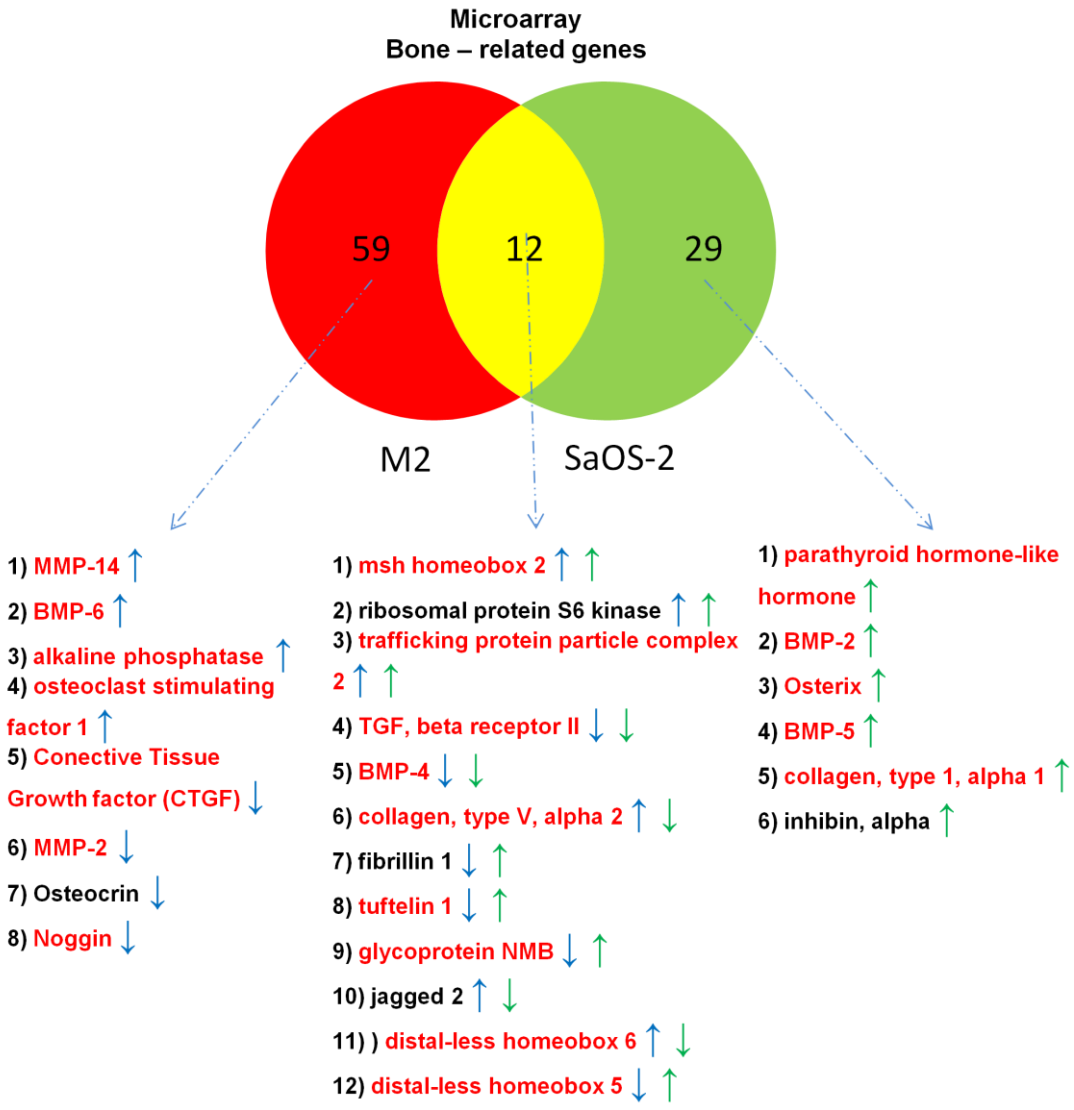


Fig. 5.4 Venn diagram showing overlap of genes differentially expressed in M2 and SaOS-2 cells, where Filamin A is absent or downregulated, respectively. The red and green circles represent the genes that changed in M2 cells and SaOS-2, respectively. The yellow zone represents the overlapped genes in both cell lines. The most representative genes involved in bone formation were selected, the genes in red fonts are the ones selected for RT-qPCR validation analysis. In front of each gene there is an arrow facing upwards or downwards, representing an up- or downregulated gene, respectively. In the overlapped genes, the blue arrows represent the changes seen in M2 cells. Green arrows represent the changes seen for the same gene in SaOS-2 cells.

Accession Number	Gene	Abr.	Function
NM_000478	alkaline phosphatase, liver/bone/kidney	ALP	Play a role in skeletal mineralization
NM_001718	bone morphogenetic protein 6	BMP-6	Induces cartilage and bone formation. Belongs to the TGF-beta family.
NM_004995	matrix metallopeptidase 14	MMP-14	Involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, differentiation.
NM_012383	osteoclast stimulating factor 1	OSF	Induces bone resorption, by secretion of factor(s) enhancing osteoclast formation and activity
NM_004530	matrix metallopeptidase 2	MMP-2	Involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development,
NM_005450	Noggin	NOG	Essential for cartilage morphogenesis and joint formation. Inhibitor of bone morphogenetic proteins (BMP) signalling
NM_001202	bone morphogenetic protein 4	BMP-4	Induces cartilage and bone formation.
NM_000393	collagen, type V, alpha 2	Col5a2	Member of group I collagen, It is a minor connective tissue component
NM_005222	distal-less homeobox 6	Distbox6	Expressed in brain and bones, and also in all skeletal structures of midgestation embryos after the first cartilage formation
NM_001024847	transforming growth factor, beta receptor II	TGB-beta	This receptor/ligand complex phosphorylates proteins, which then enter the nucleus and regulate the transcription of a subset of genes related to cell proliferation
NM_152860	Sp7 transcription factor, Osterix	OSX	Transcriptional activator essential for osteoblast differentiation.
NM_001200	bone morphogenetic protein 2	BMP-2	Induces cartilage and bone formation, online information: Bone morphogenetic protein 2 entry, similarity: Belongs to the TGF-beta family.
NM_021073	bone morphogenetic protein 5	BMP-5	Induces cartilage and bone formation., online information: Bone morphogenetic protein 5 entry, similarity: Belongs to the TGF-beta family
NM_005221	distal-less homeobox 5	Dbox5	In day 12.5 embryos, expressed in the brain and bones, and also in all skeletal structures of midgestation embryos after the first cartilage formation.
NM_001005340	glycoprotein (transmembrane) nmb	GNMB	Is expressed in osteoblast cultures at all stages of differentiation, it is also called Osteoactivin
NM_002449	msh homeobox 2	Msx2	Regulates bone formation. Plays a central role in preventing ligaments and tendons from mineralizing, in part by suppressing Runx2/Osf2 transcriptional activity
NM_198965	parathyroid hormone-like hormone	PTHorm	Regulates endochondral bone development
NM_001011658	trafficking protein particle complex 2;	TPPC2	Defects in TRAPPC2 are the cause of spondyloepiphyseal dysplasia tardia, a recessive disorder of endochondral bone formation.
NM_020127	tuftelin 1	Tuftelin	Involved in the mineralization and structural organisation of enamel.
NM_001901	Conective Tissue Growth Factor	CTGF	Major connective tissue mitoattractant secreted by vascular endothelial cells. Promotes proliferation and differentiation of chondrocytes.

Table 5.0 Selected bone related genes derived from gene microarray analysis. All the information shown in the table, including the gene function was extracted from www.genecards.org (Safran et al, 2010).

5.5 Gene microarray validation by RT-qPCR.

To validate the results obtained from the microarray, a total of 20 up or downregulated genes (see Table 5.1) were individually validated by specific RT-qPCR assays in M2 and SaOS-2 cells (Fig. 5.5). Once RT-qPCR was performed, the data was analysed and cross-compared with the microarray data. If the RT-qPCRdata matched the same criteria seen by microarray analysis then that gene was considered as a successfully validated gene.

By PCR analysis in M2 cells, the upregulated genes were: *MMP-2, MMP-13, MMP-14, BMP-4, BMP-5, BMP-6, alkaline phosphatase, msh homeobox 2, collagen type V, distal-less homeobox 6, Noggin and Osterix*. The downregulated genes were: *Conective Tissue Growth factor (CTGF), TGF- beta receptor II and Filamin A. Conversely, osteoclast stimulating factor 1, trafficking protein particle complex 2, BMP-2, Distal less homeobox5, parathyroid hormone-like hormone, glycoprotein NMB and tuftelin* showed no significant changes (Fig. 5.5 A). From M2 cell analysis, 8 genes were successfully validated by RT-qPCR: Alkaline phosphatase, BMP-6, MMP-14, Collagen type V alpha 2, distal-less homeobox 6, TGF-beta receptor II and msh homeobox 2 (see table 5.1, orange cells).

In Filamin A-knockdown SaOS-2 cells, the upregulated genes in the RT-qPCR analysis were: *MMP-2, MMP-13, MMP-14, BMP-5, BMP-6, alkaline phosphatase, msh homeobox 2, collagen type V, distal-less homeobox 6, Noggin and Osterix, Conective Tissue Growth factor (CTGF), TGF- beta receptor II, osteoclast stimulating factor 1, trafficking protein particle complex 2, BMP-2, glycoprotein NMB and tuftelin. Parathyroid hormone-like hormone, BMP-4* and *Distal less homeobox5* showed no

significant changes. However, no downregulated genes, except from Filamin A, were present in the analysis (Fig. 5.5 B). In total, 8 genes were successfully validated by RT-qPCR: *Sp7 transcription factor or Osterix, BMP-2, BMP-5, distal-less homeobox 5, glycoprotein NMB* and *msh homeobox 2* (see table 5.2, orange cells).

In summary, by gene microarray analysis and RT-qPCR, it can be suggested that the downregulation of Filamin A can influence a set of genes involved in skeletal formation and osteoblastic differentiation. Whether the changes in gene expression patterns are direct or indirect, it seems that Filamin A could play an important role in bone biology.

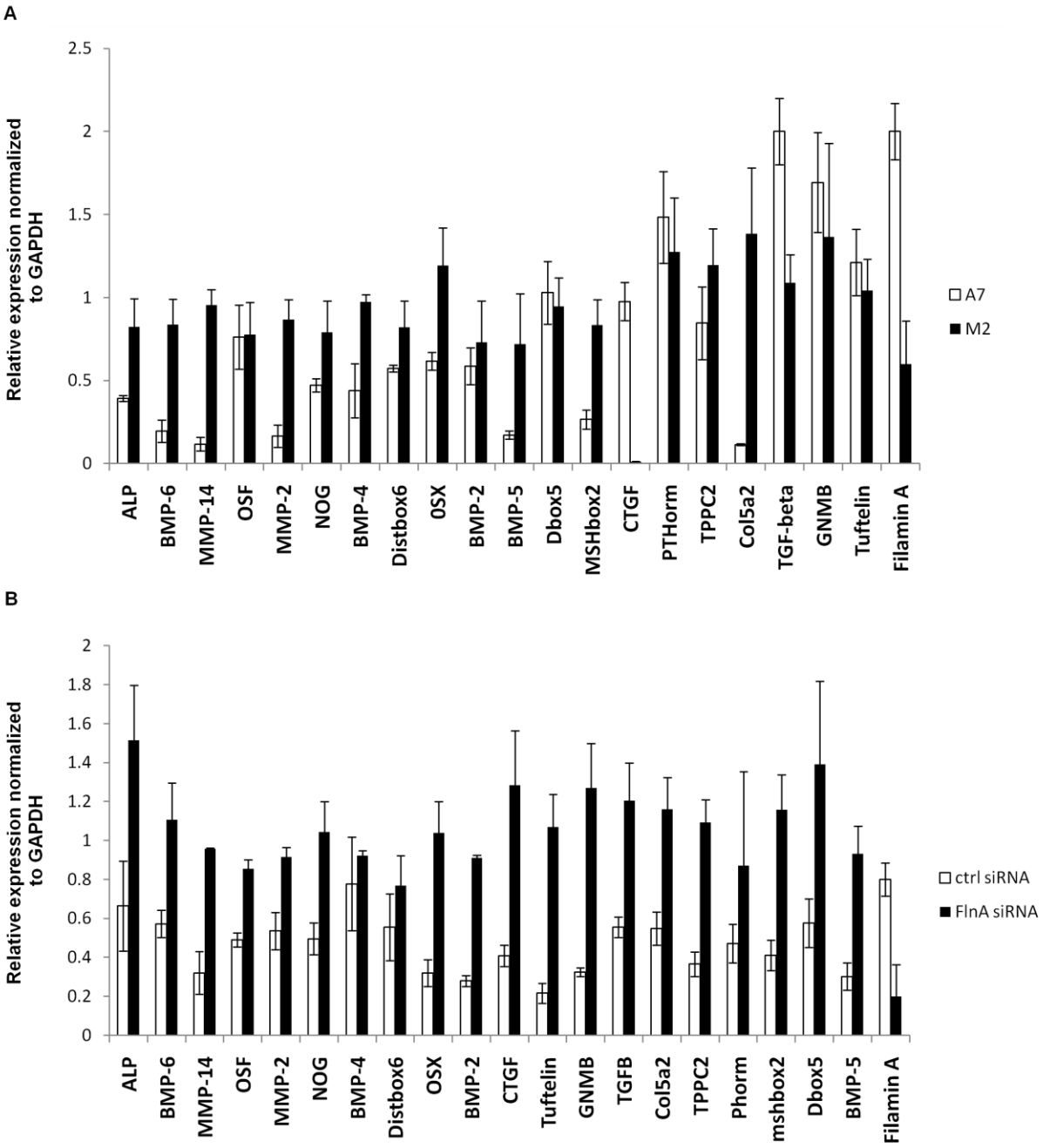


Fig. 5.5 RT-qPCR validation of genes derived from microarray analysis. Quantitative RT-qPCR validation of some genes shown to be differentially regulated by the microarray experiment in the Filamin A negative M2 and the Filamin A-positive A7 cells (A), and in the Filamin A downregulated SaOS-2 cells and its control (B). GAPDH was used as a housekeeping gene control.

		M2 Microarray			
Gene	**Abbreviation**	Downr	Upreg	RT-qPCR	PASS
alkaline phosphatase, liver/bone/kidney	ALP			UPREGULATED	YES
bone morphogenetic protein 6	BMP-6			UPREGULATED	YES
matrix metallopeptidase 14	MMP-14			UPREGULATED	YES
osteoclast stimulating factor 1	OSF			NO CHANGES	NO
matrix metallopeptidase 2	MMP-2			UPREGULATED	NO
Noggin	NOG			UPREGULATED	NO
bone morphogenetic protein 4	BMP-4			UPREGULATED	NO
collagen, type V, alpha 2	Col5a2			UPREGULATED	YES
distal-less homeobox 6	Distbox6			UPREGULATED	YES
transforming growth factor, beta receptor II	TGB-beta			DOWNREGULATED	YES
Sp7 transcription factor, Osterix	OSX			UPREGULATED	-----
bone morphogenetic protein 2	BMP-2			NO CHANGES	-----
bone morphogenetic protein 5	BMP-5			UPREGULATED	-----
distal-less homeobox 5	Dbox5			NO CHANGES	NO
glycoprotein (transmembrane) nmb	GNMB			NO CHANGES	NO
msh homeobox 2	MSHbox2			UPREGULATED	YES
parathyroid hormone-like hormone	PTHorm			NO CHANGES	------
trafficking protein particle complex 2;	TPPC2			NO CHANGES	NO
tuftelin 1	Tuftelin			NO CHANGES	NO
Conective Tissue Growth Factor	CTGF			DOWNREGULATED	YES

Table 5.1 PCR validation results for M2 cells.

		SAOS-2 ARRAY			
Gene	**Abbreviation**	Downr	Upr	PCR result	PASS
alkaline phosphatase, liver/bone/kidney	ALP			UPREGULATED	--------
bone morphogenetic protein 6	BMP-6			UPREGULATED	--------
matrix metallopeptidase 14	MMP-14			UPREGULATED	--------
osteoclast stimulating factor 1	OSF			UPREGULATED	--------
matrix metallopeptidase 2	MMP-2			UPREGULATED	--------
Noggin	NOG			UPREGULATED	--------
bone morphogenetic protein 4	BMP-4			NO CHANGES	NO
collagen, type V, alpha 2	Col5a2			UPREGULATED	NO
distal-less homeobox 6	Distbox6			NO CHANGES	NO
transforming growth factor, beta receptor II	TGB-beta			UPREGULATED	NO
Sp7 transcription factor, Osterix	OSX			UPREGULATED	YES
bone morphogenetic protein 2	BMP-2			UPREGULATED	YES
bone morphogenetic protein 5	BMP-5			UPREGULATED	YES
distal-less homeobox 5	Dbox5			UPREGULATED	YES
glycoprotein (transmembrane) nmb	GNMB			UPREGULATED	YES
msh homeobox 2	MSHbox2			UPREGULATED	YES
parathyroid hormone-like hormone	PTHorm			NO CHANGES	NO
trafficking protein particle complex 2;	TPPC2			UPREGULATED	YES
tuftelin 1	Tuftelin			UPREGULATED	YES
Conective Tissue Growth Factor	CTGF			UPREGULATED	--------

Table 5.2. PCR validation results for SaOS-2 cells.

5.6 Discussion

Microarray experiments were designed in order to analyse changes in gene expression in two different cell types in which Filamin A was either depleted or not present. First, the Filamin A-negative M2 cell line versus its control, the Filamin A-positive A7 cell line was analysed. Secondly, the changes in gene expression in Filamin A knockdown SaOS-2 cells induced by Filamin A-specific siRNA versus its control was also studied. I demonstrated that there are significant differences in the gene expression profiles of Filamin A-negative and Filamin A-positive cells. Moreover, a subset of the differentially expressed genes was known to be involved in bone biology.

5.6.1. Experimental design

The design utilized in this work was to replicate the microarray experiment in triplicate in order to gather more confident information on each of the genes examined on the microarrays. In this case, replication involved biological replicates (samples prepared in separate but using the same conditions) but not technical replicates, which would prove especially useful in determining the variability of each gene. However, in this case of experiments, using technical replicates makes the study more expensive and it does not give too much extra information on the changes of expression.

5.6.2 Analysis of the microarray data

The development of methods for the analysis of microarray data is important to gather out the best information of the experiments. The methods for analyzing microarrays are diverse and in constant development and improvement (Allison et al, 2006; Wu, 2001). In this thesis, the data was organised as a ranked list according to the fold change. The

fold change was given by 1) comparing the mean of the triplicates between the Filamin A depleted and normal samples and 2) by the two statistical approaches to assess significance (p value, which indicates which genes are false-positive and the q value, which indicates how significantly the gene expression changes). This approach enabled the search for genes that, on average, performed consistently well across all the triplicates. This method still permitted the examination of the ranked genes from any of the individual lists. The microarray analysis provides a ranked list of the changes in gene expression based on statistical significance. With regard to the ranked list, it is difficult to determine where the cut-off is between the real and false positives. The amount of genes differentially expressed from the microarray analysis according to a cut off of from -1.25 to 1.25 fold change is presented in Fig. 5.0. However, is likely that there are other significantly expressed genes that are found outside this analysis. Different methods of gene expression analysis (RT-qPCR or Northern Blots) are performed in order to identify where the cut-off exists in the array data and to confirm the differential expression of each gene (Jayapal & Melendez, 2006; Provenzano & Mocellin, 2007). The information obtained from these other methods could then be of help to determine at which extent the analysis start to disagree with quantitative methods of gene expression analysis. However, given the amount of genes differentially expressed, this approach is time consumable and technically difficult as it would need a big amount of mRNA to perform all the validations. Because of that, the selection of bone related genes was performed first and then the pertinent validation by RT-qPCR was carried out.

5.6.3 Selection of known specific bone related genes

Using the list of differentially expressed genes gathered from the microarray, a list of genes of interest was built based on known function and gene ontology by using DAVID database application. The genes associated with bone biology included a number of genes that have been previously shown to induce bone formation or calcification, skeletal development and osteoblastic differentiation. A group of genes which covered all the genes related to bone biology were represented in heat maps and are shown in Fig. 5.3 and 5.4. To identify significant bone related genes, a comparative analysis of the microarray from the two different cell lines M2 and SaOS-2 was performed. Some of those genes overlapped in both microarrays indicating reproducibility and consistency in the data (see Fig. 5.5). The purpose of performing a microarray in both cell lines was to gather more information about changes induced by Filamin A downregulation. However, it could be important to correlate the results with another osteoblastic cell line in order to look for changes in osteal cells only. Furthermore, it cannot be rule out the fact that the expression profiles on bone related genes between the samples are different since the fact that M2 cells is a melanoma cell line and SaOS-2 is an osteosarcoma cell line.

5.6.4 The use of RT-qPCR to confirm differential expression

The interpretation of a relevant microarray data is subject to the differential expression observed in the microarray, which in turn could be indicative of the actual changes in gene expression, and also is subject to whether the differentially expressed genes are indeed significant. In order to gain a better idea of how many genes where actually behaving in the same way as the microarray data reflected, RT-qPCR experiments were

performed. 20 statistically significant genes identified by the microarray analysis were examined using RT-qPCR to confirm their differential expression in M2 and SaOS-2 cell lines, along with the controls. The initial PCR validations confirmed that 8 out of 20 genes derived from the microarray analysis displayed differential expression between the Filamin A-negative M2 and the Filamin A-positive A7. These genes are: *alkaline phosphatase, bone morphogenetic protein 6, matrix metallopeptidase 14, collagen, type V, alpha 2, distal-less homeobox 6, transforming growth factor, beta receptor II and Conective Tissue Growth Factor*. Similarly, 8 out of 20 genes displayed the same differential expression between Filamin A knockdown SaOS-2 and its control. These genes are: *Sp7 transcription factor/ Osterix, bone morphogenetic protein 2, bone morphogenetic protein 5, distal-less homeobox 5, glycoprotein (transmembrane) nmb, msh homeobox 2, trafficking protein particle complex 2 and tuftelin 1.* It is important to note that more than a half of the genes tested did not show any changes by RT-qPCR. In this regard one explanation is that those genes were located near to the limits of the cut off values derived from the fold change in the microarray data. However, the genes with a relatively small change or no change in gene expression are worthy of further investigation in another context to examine whether Filamin A fluctuations can alter both mRNA or protein levels in the cell. Another explanation for the discrepancies between RT-qPCR experiments and microarray data could be the small variations between culture conditions, suggesting that some genes are regulated by subtle changes in growth conditions (for example a different batch of FBS) that occurred between experiments.

5.6.5 Role of Filamin A in regulating bone genes

In this section it will be discussed the interplay between some important bone related genes that were derived from microarray and PCR validations. The genes discussed below have shown to be absolutely important to promote bone formation. These genes are: *Sp7 transcription factor (Osterix), bone morphogenetic protein 2 and msh homeobox 2.* Since Filamin A downregulation affected the genes mentioned above, it is important to discuss how this genes work together to promote bone formation. Osterix transcription factor or also called Sp7, a member of the Sp1 transcription factor family, has been show to play an essential role in bone formation by allowing the development of osteoblasts (Lian et al, 2006; Maruyama & Komori, 2007). Although Osterix has been shown to be induced by Runx2 and BMP2, the regulation and function of Osterix during osteoblast differentiation, is not well understood. One of the functions of Osterix is the induction of alkaline phosphatase activity and osteocalcin expression to stimulate calcification (Matsubara et al, 2008). The homeobox gene Msx2, which is up-regulated by BMP2, is able to regulate osteoblastic differentiation by repressing Alkaline phosphatase promoter activity by antagonizing the stimulatory effect of DLX5 (Cho et al, 2009).

In this thesis, it has been shown by microarray analysis that Filamin A is able to regulate the genes mentioned above. At the moment it can be proposed that the upregulation of these genes might be due to the increase of BMP-2 pathway activity which in part can be enhanced by the Smad proteins. Bone morphogenetic proteins (BMPs) are a family of proteins that induce bone and cartilage formation *in vivo*. They play pivotal roles during early embryonic development and tissue morphogenesis

(Katagiri et al, 1994; Matsubara et al, 2008; Phimphilai et al, 2006). Furthermore, a set of Smad proteins, involved in the BMP signalling pathway, have been reported to interact with Filamin A by yeast two-hybrid screening (Sasaki et al, 2001). In that study it has also been shown that TGF-β signalling was defective in the Filamin A-negative human melanoma cells M2 compared with a Filamin A-transfected melanoma sub line A7. Therefore the changes in gene expression seen in this work can be due to the downregulation of Filamin A which in turn could induce the Smad proteins to go to the nucleus and activate gene expression. Another option is that Filamin A could transcriptionaly regulate any of the genes previously mentioned. More studies have to be performed in order to gain a better idea of the role of Filamin A in the regulation of bone formation and osteoblastic differentiation. The elucidation of changes in gene expression by the downregulation of Filamin A is an important step trough understanding the role of Filamin A in bone formation since mutations of Filamin A in humans reflects bone malformation syndromes.

6.0 General Discussion and Future Perspectives

6.1 Role of Filamin A and Runx2 in bone formation.

The project conducted in this work shows that Filamin A, an actin binding protein which has been mostly associated to the cytoskeltal structure can influence the activity of the well known osteogenic transcription factor Runx2. This idea arose from two lines of evidence. The first line of evidence suggests that mutations in Filamin A somehow alter the physiology of bone formation which can cause congenital skeletal malformations as otopalatodigital syndrome (OPD), frontometaphyseal dysplasia (FMD) and Melnick–Needles syndrome (MNS) (Foley et al, 2010; Robertson et al, 2003). Over the years Filamin A mutations have been mapped and reported as patient case reports (Marino-Enriquez et al, 2007; Robertson et al, 2003; Sun et al, 2010; Wu, 2001). Interestingly, there is a considerable amount of reported mutations which are located in different areas of the gene and they are spanned in different portions of Filamin A. This can indicate that not only one segment of Filamin A is involved in maintaining bone homeostasis. Furthermore two mouse models of Filamin A deficiency have been reported in the past years (Feng et al, 2006; Hart et al, 2006). Their phenotypes include mild skeletal abnormalities in female heterozygous mice, and skeletal and palate defects in homozygous male mice, between others. Whether these skeletal disorders seen in both human and mouse organism are caused by structural perturbations of Filamin A, by alterations of interactions with other proteins and signalling pathways, or by directly influencing the transcriptional activity involved in bone biology has remained poorly understood and is worthy of investigation. The second line of evidence shows that Filamin A is able to complex with the Runx family heterodimerisation partner CBFβ (Yoshida, 2005). The authors showed that Filamin A

directly interacts and retains CBFβ in the cytoplasm which in turn affects its complex with the transcription factor Runx1. They showed that when Filamin A was not present in the system, cytoplasmic CBFβ can translocate into the nucleus (Yoshida, 2005). Previous studies in our group have found the interaction between Filamin A and CBFβ by two yeast hybrid system, GST pulldowns and transfection studies. Furthermore, it has been determined, mainly by GST-pulldowns, that not only CFBβ can interact directly with Filamin A but also the transcription factor Runx2 (Yeng Yang Tang, unpublished result). Taken together the two lines of evidence discussed above, it was determined to perform further studies which aimed to explore the possible interaction between Filamin A and Runx2 and its biological role in osteal cells.

6.2 Filamin A interaction with Runx2 is likely to happen in the nucleus.

In this study, it has been shown for the first time that endogenous Filamin A and Runx2 proteins can indeed interact in osteal cells. Since these two proteins have profound effects in skeletal formation the subcellular localisation of both proteins was studied. Chapter three shows that Filamin A, as well as Runx2 are both co-localised to the nucleus. It is important to mention that full length Filamin A was detected in the nuclear fraction. There is some controversy about whether full length or a cleaved portion of Filamin A is able to enter to the nucleus (Bedolla et al, 2009; Berry et al, 2005; Wang et al, 2007). To date, there is no a definitive mechanism that might explain the nuclear import of Filamin A. Reports have suggested that a fragment of Filamin A can associate with proteins that bear a nuclear localisation signal, which in turn can carry over Filamin A to the nucleus (Loy et al, 2003). This might be the case for Runx2, a nuclear transcription factor known to shuttle between the nucleus and cytoplasm.

Therefore, it may be a possibility that Runx2 or Runx2/ CFBβ could be associated with Filamin A prior to be transported to the nucleus. Another possibility is that Filamin A could enter to the nucleus without any interaction with nuclear proteins. One important fact is that, due to the size of full length Filamin A (280 kDa) it is unlikely (but not impossible) that it can enter through the nuclear pore complex. The nuclear pore complex allows the entering to small molecules, metabolites and ions, but acts as a highly efficient molecular filter for macromolecules; this being its main function (Wente & Rout, 2010). Furthermore, one study has revealed that the nuclear pore allows the passive diffusion of proteins with size of 90 to 110 kDa (Wang & Brattain, 2007). The authors, however, do not exclude the possibility that the nuclear pore may allow the diffusion of proteins that are even larger than 110 kDa. Taken this into account the nuclear localisation of cleaved Filamin A fragments (100-110 kDa) might be explained by nuclear pore passive translocation but it cannot be explain for the full length Filamin A which is almost a third bigger than the proposed protein limit size.

It is well known that during prophase the nuclear envelope dissembled allowing the condensed chromosomes to attach to the cytoplasmic microtubules (Larijani & Poccia, 2009). Therefore, it might hypothesized that during mitosis Filamin A associates to the chromosomal structures and thus Filamin A remains attached to them until the completion of mitosis (telophase), where two new nuclei are formed. Thus, it may be possible that nuclear full length Filamin A is the result of this protein to be localised and retained into the subsequent nuclear reorganisation. Further experimental approaches need to be conducted in order to gain a better knowledge of how full length Filamin A is able to locate in the nuclear compartment.

6.3 Biological implications of Filamin A/Runx2 complex.

In chapter 3, it has been demonstrated that Filamin A is recruited to the promoter of the bone matrix-degrading enzyme, matrix metalloproteinase-13 (MMP-13), and suppresses its Runx2-dependent expression. Also it is shown that during differentiation Filamin A is downregulated and MMP-13 is upregulated. This finding supports the idea that Filamin A is a negative regulator of MMP-13 and demonstrates that Filamin A directly regulates the function of the transcription factor Runx2.

In agreement with the fact that Filamin A can influence gene expression, in this case, by direct complex with Runx2, it has been confirmed that Filamin A also interacts with different transcription factors: Androgen Receptor, Smads, FOXC1 and P73α (Berry et al, 2005; Kim et al, 2007; Loy et al, 2003; Sasaki et al, 2001). In this section it will be discussed briefly the mechanism by which Filamin A can influence the activity of these transcription factors to impact gene expression: Full length Filamin A can be cleaved *in vivo* by calpains to produce a 180 kDa N-terminal fragment and a 100 kDa C-terminal fragment. The C-terminal 100 kDa fragment localises to the nucleus to inhibit the activity of the transcription factors FOXC1 (Berry et al, 2005) and the Androgen Receptor (Loy et al, 2003). The C-terminal repeats 16-24, which is around 100 kDa was found to repress the androgen receptor transactivation by competing with the co-activator TIF2 (transcriptional intermediary factor 2) (Loy et al, 2003). Also, FOXC1 interacts with filamin A (Berry et al, 2005). In A7 melanoma cells, FOXC1 is unable to activate transcription. This inhibition is mediated through an interaction between FOXC1 and the homeodomain protein PBX1a. In addition, efficient nuclear and subnuclear localisation of PBX1 is mediated by Filamin A. The inhibitory activity of

Filamin A on FOXC1 may contribute to the pathogenesis of Filamin A-linked skeletal disorders (Berry et al, 2005).

A set of Smad proteins, involved in the Transforming Growth Factor-β (TGF-β) signalling pathway, have been reported to interact with Filamin A by yeast two-hybrid screening. It has been shown that TGF-β signalling was defective in the Filamin A-negative human melanoma cells M2 compared with a Filamin A-transfected subline A7. M2 cells restored TGF- β responsiveness following transient transfection of full-length Filamin A encoding vector (Sasaki et al, 2001).

It has been suggested that that the transcription factor p73α is sequestered in the cytoplasm by Filamin A *in vivo*, thereby inhibiting its transcriptional activity (Kim et al, 2007). p73α has been shown to be remained in the cytoplasm in A7 cells, but localised in the nucleus of filamin A-negative M2 cells. Kim and collegues also reported that Filamin A specifically interacts with the p73α C-terminus and its deletion resulted in nuclear localisation (Kim et al, 2007).

Results gathered in this thesis acknowledge the capability of Filamin A to localise to the nucleus and to interact directly with the transcription factor Runx2. The ability of Filamin A to bind and repress the MMP-13 promoter via Runx2 provides the first evidence that connects directly Filamin A as an important regulator of gene epression.

The downregulation of Filamin A might be an important step to allow the activation of Runx2 target genes to continue the differentiation program. Therefore, any changes on Filamin A levels, Filamin A malfunction, or gain of function, could provoke defects in the transcriptional regulation during differentiation. In this regard, a proteomics analysis in human immortalized pre-osteoblasts, which are able to differentiate and

mineralize into mature osteoblast showed that downregulation of Filamin A is detected at day 19 in mineralizing osteoblast versus the pre-mineralization osteoblastic period (Alves et al, 2010), which suggests that Filamin A levels have to be reduced to favour, in this case, differentiation. This finding correlates with the results showed in this thesis in which Filamin A is downregulated during the differentiation process. In this work, MMP-13 gene is shown to be regulated by Filamin A via Runx2 by associating to its promoter. Nevertheless, it will be important to explore closely more Runx2-target genes, under different experimental conditions, in which Filamin A may possibly regulate its gene transcriptional activity. Finally, the association with transcription factors with this versatile protein is particularly interesting as it suggests that Filamin A plays a pivotal role in linking changes in the cytoskeleton to the regulation of gene expression.

6.4 Actin-binding proteins in the nucleus

Actin is an abundant cytoskeletal protein which participates in many important cellular processes including muscle contraction, cell division and cytokinesis, cell signalling, and maintenance of cell shape by the cytoskeletal component (Holmes et al, 1990; Khaitlina, 2001; Oda & Maeda, 2010). Actin has been found in the nucleus and it has been shown to have important roles in chromatin remodelling, transcriptional regulation, RNA processing, and nuclear export (de Lanerolle et al, 2005; Philimonenko et al, 2004). Actin also regulates RNA polymerases I, II, and III (Kukalev et al, 2005; Philimonenko et al, 2004; Ye et al, 2008). Nonetheless, there is controversy about nuclear actin localisation, for example, there is absence of nuclear actin labeling by using phalloidin, which strongly and specifically binds to actin. However, a pre-

treatment of nuclear preparations with two different endonucleases to remove a substantial amount of chromosomal DNA uncovered the presence of nuclear actin for both antibody and phalloidin detection (Sauman & Berry, 1994). A number of actin-binding and actin-related proteins have been identified in the nucleus where they have been shown to participate in transcriptional regulation, splicing, chromatin remodelling and DNA repair (Gettemans et al, 2005; Olave et al, 2002). However, it is largely unknown if these proteins can function on their own or by association with actin, in part because the nuclear localisation of actin has been controversial and there is not a total agreement of whether fibrillar or monomeric actin is located in the nucleus. As the actin binding protein, Filamin A, has been found in the nuclear compartment, there is the question if this protein can interact with nuclear actin in order to perform gene regulation. Whether or not actin is important for Filamin nuclear function remains to be unknown. More studies have to be carried out in order to answer this question.

6.5 The nucleolar localisation of Filamin A

In this project, a careful look into the localisation of Filamin A in the nucleus has revealed that Filamin A can also be aggregated in the nucleolus, an important nuclear architecture domain. Chapter 4 of this thesis presented compelling evidence that shows that Filamin A is located in the nucleolar compartment in different cell types and in primary cultured cells. Antibodies against nucleolar proteins were used in order to delineate the localisation of Filamin A in this compartment, suggesting that Filamin A is located in the granular component and the fibrillar centre of the nucleolus. Also, a nucleolar localisation signal has been detected in the carboxy terminal of Filamin, which could support its nucleolar retention.

The nucleolar localisation of Filamin A reported in this thesis is supported by two different research groups. Nucleolar localisation of Filamin A has been reported in the nucleolar proteome database (Angus Lamond et al, personal communication). Furthermore, it has been shown that the immunogold labeling of Filamin A in ultrathin sections of HeLa cells is intense and appears as a dense mesh throughout the whole nucleus, including nucleoli. The authors reported that Filamin A is apparently present in all three nucleolar components (Dingova et al, 2009). Further experiments need to confirm that the possible nucleolar localisation signal in Filamin A is indeed a nucleolar localisation signal. This could be achieved by mutation of the nucleolar localisation signal located in Filamin A. By doing this, it can be speculated that without the nucleolar localisation signal, Filamin A might reduce its nucleolar localisation and therefore its rRNA regulation activity.

6.6 Biological significance of nucleolar Filamin A

In this work it has been shown that the rDNA promoter can be regulated by Filamin A and Runx2 by studying the activity of the rDNA promoter which had its Runx2 site mutated. Also, it has been shown that the absence of Filamin A favours rRNA transcription, suggesting that Filamin A is involved in the regulation of ribosomal DNA expression by RNA polymerase I. To our knowledge this is the first report in which Filamin A is implicated in the regulation of ribosomal DNA expression. Ribosomal DNA (rDNA) codes for ribosomal RNA (Hozak et al, 1994). The nucleolus is the factory in which rRNAs are synthesized, processed and assembled with ribosomal proteins to form the subunits of the ribosomes (Sirri et al, 2008). The transcription of ribosomal genes is driven by the RNA polymerase I machinery (Carmo-Fonseca et al,

2000; Russell & Zomerdijk, 2006). In this context, nucleolar Filamin A is suggested to play an important role in regulating the expression of ribosomal genes, in part because it has been shown in this thesis that Filamin A is located in the fibrillar centre and in the granular component. Importantly, Filamin A is segregated from the nucleolus when the drug actinomycin D is used to inhibit specifically rRNA synthesis by RNA polymerase I. Finally the results found by Dr. WengSheng Deng in our group, in which Filamin A is located at the promoter regions of rDNA add another strong evidence to conceive Filamin A as an important regulator of gene expression. In conclusion, the biological importance of nucleolar Filamin A, its distribution during the cell cycle, the regulation of rDNA genes and the mechanisms by which Filamin A can complex to the promoter regions to repress ribosomal gene expression have to be addressed in more detail in further experimental designs.

6.7 The microarray approach to determine gene expression patterns in absence of Filamin A

With the development of microarray technology, now it has been able to obtain broad and accurate estimates of global gene expression levels. One of the aims in this project was to analyse the differential gene expression related to bone biology in the absence of Filamin A. These experiments therefore could provide initial clues into the molecular consequences of the absence of Filamin A in osteal cells. The data gathered in chapter 5 has shown that Filamin A knockdown induced the downregulation of a considerable amount of genes in two different cell lines. The first cell line was a melanoma cell line (M2) that naturally does not express Filamin A and the second, is an osteosarcoma cell line denominated SaOS-2 which was subjected to a downregulation of its Filamin A

basal levels. It is important to mention that M2 cells were used in order to obtain data related to bone differential gene expression, even when this cell is not derived from the osteogenic lineage. The reason of why this cell line was chosen to be analysed is because in this model Filamin A has not been present from the very moment the cell line was developed (Cunningham et al, 1992). It has been shown and broadly discussed that transient changes in a specific gene could not provide a real estimate of the changes in gene expression (Tan et al, 2003). This is because the cell has not completely "adapted" to its new biological activity. Nonetheless, transient alterations in gene expression have proven to be useful to detect significant and important changes (Wu, 2001). Therefore, M2 analysis could be of interest in detecting changes that otherwise could not been appreciated in transient Filamin A knockdown SaOS-2 cells. One important challenge to be accomplished in this regard is the creation of an inducible Filamin A expression cell or a stable knockdown-Filamin A osteoblastic cell line. This would represent a unique model which could provide a better understanding about Filamin A biology related to bone regulation.

The analysis of the microarray data from the two cell lines in this study has revealed that absence of Filamin A induced the upregulation and downregulation of genes that are profoundly involved in bone formation. From those genes, 20 candidate genes involved in bone biology were selected and afterwards subjected to validation by RT-qPCR. The osteoblasts differentiation is basically orchestrated by the activation of bone related transcription factors (Alves et al, 2010; Bourne et al, 2004; Hong et al, 2010; Maruyama & Komori, 2007; Nakashima & de Crombrugghe, 2003). Runx2 and Osterix have been shown as master controllers of the osteoblastic lineage and the absence of

either one results in a complete lack of bone mineralization (Maruyama & Komori, 2007; Nakashima & de Crombrugghe, 2003; Prince et al, 2001; Schroeder et al, 2005). Also, other transcription factors have been shown to regulate osteoblast function, including homeobox proteins: MSX1, MSX2, DLX3, and DLX5, among others (Barnes et al, 2003; Matsubara et al, 2008; Shum & Nuckolls, 2002). In this work it has been shown for the first time that important transcription factors and proteins required for bone formation displayed differential gene expression when Filamin A is absent: This genes are: Sp7 transcription factor (Osterix), bone morphogenetic protein 2, Distal less homeobox 5 (DLX5) and Msx2.

6.8 An integrated view of Filamin A roles during osteoblast differentiation

The scheme, presented in Fig. 6.0, highlights the roles of some of the most important proteins found to be differentially regulated by Filamin A changes by microarray. These proteins have essential roles in regulating osteoblastic differentiation. In the following paragraph an integrated view of the possible roles of Filamin A during osteoblastic formation is presented, according to the results gathered in this thesis. Osteoblasts differentiate from their mesenchymal precursors in a complex process that is orchestrated by the timely activation of specific transcription factors that regulate the expression of other genes and thus determine the osteoblast specific characteristics (Barnes et al, 2003; Ducy & Karsenty, 1998; Franceschi & Xiao, 2003; Jensen et al, 2010; Lian & Stein, 2003; Lian et al, 2006). Runx2 and OSX have been identified as master regulators of the osteoblastic lineage. MSX2 and DLX5 are members of the homeobox family and they are also important in the regulation of osteoblastic differentiation (Komori, 2006). The downregulation of Filamin A has shown that these molecules are upregulated (Fig. 6.0). This evidence correlates with the downregulation of Filamin A seen in differentiating osteoblast, which would allow these transcription factors to be upregulated and thus fulfil the differentiation program. During osteoblastic differentiation process, signalling pathways are deeply involved in regulating gene expression, as is the case for the BMP-2 signalling pathway, which has shown to be influenced by cytoskeletal Filamin A and nuclear Filamin A translocation (Sasaki et al, 2001). The interaction between Runx2 and Filamin A could be very important in regulating Runx2 related gene expression. In this thesis, it has been demonstrated that Filamin A regulates MMP-13 by complex in its promoter with Runx2. Changes in

Filamin A levels that could impact either the cytoskeletal architecture or the subcellular localisation of Filamin A (i.e. nuclear or nucleolar) may be important in the regulation of osteoblastic process and skeletal development. In this regard, the regulation of ribosomal rRNA expression may play specific roles by regulating the protein synthesis required for differentiation or bone homeostasis. Further studies should be pointed to determine if Filamin A levels can fluctuate in specific stages, from stem cells to fully differentiated osteoblast. By doing this it could be explained in some extent the clinical manifestation of Filamin A bone related diseases.

The results gathered in this thesis show novel findings in which Filamin A, a typical cytoskeletal protein, can also perform different functions on the cell such as genetic regulation by associating with the promoter of certain genes. Also, it seems that changes in Filamin A expression are important in order to trigger different cellular mechanisms to support osteoblastic differentiation. This thesis therefore uncovers new exciting functions of Filamin A that might not to be only important for bone biology but for general cell biology itself.

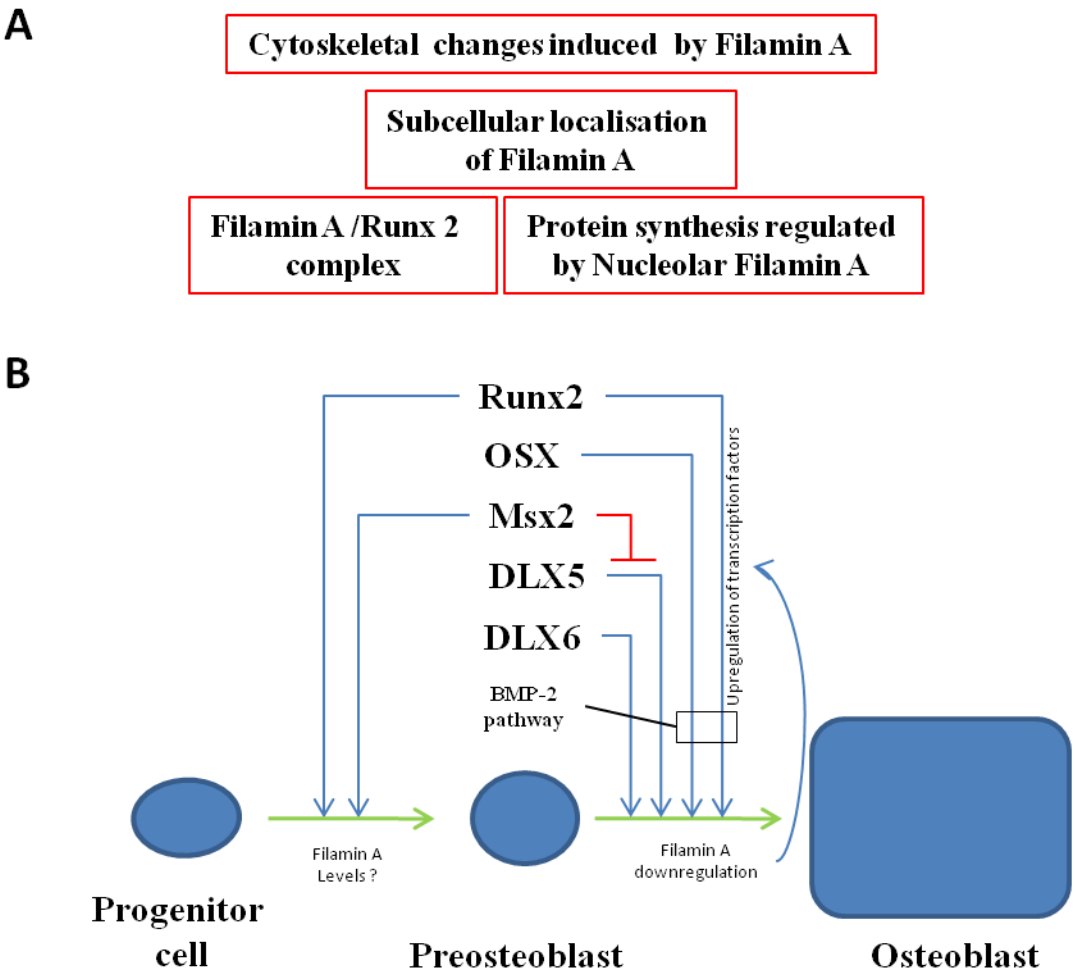


Fig. 6.0 An integrated scheme of Filamin A roles during osteoblast differentiation. (A) The possible roles of Filamin A during osteoblastic differentiation are also shown and they include the Filamin A/Runx2 complex, the subcellular Filamin A localisation and the changes in Filamin A basal levels. (B) Schematic representation of the genes involved in bone regulation gathered by microarray analysis except Runx2, which did not show differential changes in Filamin A downregulated cells.

7.0 References

Abrass CK, Hansen KM (2010) Insulin-like growth factor-binding protein-5-induced laminin gamma1 transcription requires filamin A. *J Biol Chem* **285**(17)**:** 12925-12934

Ahmad Y, Boisvert FM, Gregor P, Cobley A, Lamond AI (2009) NOPdb: Nucleolar Proteome Database--2008 update. *Nucleic Acids Res* **37**(Database issue)**:** D181-184

Aimes RT, Quigley JP (1995) Matrix metalloproteinase-2 is an interstitial collagenase. Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native type I collagen generating the specific 3/4- and 1/4-length fragments. *J Biol Chem* **270**(11)**:** 5872-5876

Akiyama H (2008) Control of chondrogenesis by the transcription factor Sox9. *Mod Rheumatol* **18**(3)**:** 213-219

Ali SA, Zaidi SK, Dobson JR, Shakoori AR, Lian JB, Stein JL, van Wijnen AJ, Stein GS (2010) Transcriptional corepressor TLE1 functions with Runx2 in epigenetic repression of ribosomal RNA genes. *Proc Natl Acad Sci U S A* **107**(9)**:** 4165-4169

Allison DB, Cui X, Page GP, Sabripour M (2006) Microarray data analysis: from disarray to consolidation and consensus. *Nat Rev Genet* **7**(1)**:** 55-65

Alves RD, Eijken M, Swagemakers S, Chiba H, Titulaer MK, Burgers PC, Luider TM, van Leeuwen JP (2010) Proteomic analysis of human osteoblastic cells: relevant proteins and functional categories for differentiation. *J Proteome Res* **9**(9)**:** 4688-4700

Anglin I, Passaniti A (2004) Runx protein signaling in human cancers. *Cancer Treat Res* **119:** 189-215

Aronson BD, Fisher AL, Blechman K, Caudy M, Gergen JP (1997) Groucho-dependent and -independent repression activities of Runt domain proteins. *Mol Cell Biol* **17**(9)**:** 5581-5587

Banerjee C, Javed A, Choi JY, Green J, Rosen V, van Wijnen AJ, Stein JL, Lian JB, Stein GS (2001) Differential regulation of the two principal Runx2/Cbfa1 n-terminal isoforms in response to bone morphogenetic protein-2 during development of the osteoblast phenotype. *Endocrinology* **142**(9)**:** 4026-4039

Barnes GL, Javed A, Waller SM, Kamal MH, Hebert KE, Hassan MQ, Bellahcene A, Van Wijnen AJ, Young MF, Lian JB, Stein GS, Gerstenfeld LC (2003) Osteoblast-related transcription factors Runx2 (Cbfa1/AML3) and MSX2 mediate the expression of

bone sialoprotein in human metastatic breast cancer cells. *Cancer Res* **63**(10)**:** 2631-2637

Bartfeld D, Shimon L, Couture GC, Rabinovich D, Frolow F, Levanon D, Groner Y, Shakked Z (2002) DNA recognition by the RUNX1 transcription factor is mediated by an allosteric transition in the RUNT domain and by DNA bending. *Structure* **10**(10)**:** 1395-1407

Beck GR, Jr., Zerler B, Moran E (2000) Phosphate is a specific signal for induction of osteopontin gene expression. *Proc Natl Acad Sci U S A* **97**(15)**:** 8352-8357

Bedolla RG, Wang Y, Asuncion A, Chamie K, Siddiqui S, Mudryj MM, Prihoda TJ, Siddiqui J, Chinnaiyan AM, Mehra R, de Vere White RW, Ghosh PM (2009) Nuclear versus cytoplasmic localization of filamin A in prostate cancer: immunohistochemical correlation with metastases. *Clin Cancer Res* **15**(3)**:** 788-796

Bee T, Swiers G, Muroi S, Pozner A, Nottingham W, Santos AC, Li PS, Taniuchi I, de Bruijn MF (2010) Nonredundant roles for Runx1 alternative promoters reflect their activity at discrete stages of developmental hematopoiesis. *Blood* **115**(15)**:** 3042-3050

Berry FB, O'Neill MA, Coca-Prados M, Walter MA (2005) FOXC1 transcriptional regulatory activity is impaired by PBX1 in a filamin A-mediated manner. *Mol Cell Biol* **25**(4)**:** 1415-1424

Bicknell LS, Morgan T, Bonafe L, Wessels MW, Bialer MG, Willems PJ, Cohn DH, Krakow D, Robertson SP (2005) Mutations in FLNB cause boomerang dysplasia. *J Med Genet* **42**(7)**:** e43

Blyth K, Cameron ER, Neil JC (2005) The RUNX genes: gain or loss of function in cancer. *Nat Rev Cancer* **5**(5)**:** 376-387

Boisvert FM, van Koningsbruggen S, Navascues J, Lamond AI (2007) The multifunctional nucleolus. *Nat Rev Mol Cell Biol* **8**(7)**:** 574-585

Bourne S, Polak JM, Hughes SP, Buttery LD (2004) Osteogenic differentiation of mouse embryonic stem cells: differential gene expression analysis by cDNA microarray and purification of osteoblasts by cadherin-11 magnetically activated cell sorting. *Tissue Eng* **10**(5-6)**:** 796-806

Bravo J, Li Z, Speck NA, Warren AJ (2001) The leukemia-associated AML1 (Runx1)--CBF beta complex functions as a DNA-induced molecular clamp. *Nat Struct Biol* **8**(4)**:** 371-378

Budde A, Grummt I (1999) p53 represses ribosomal gene transcription. *Oncogene* **18**(4)**:** 1119-1124

Carmo-Fonseca M, Mendes-Soares L, Campos I (2000) To be or not to be in the nucleolus. *Nat Cell Biol* **2**(6)**:** E107-112

Castano E, Philimonenko VV, Kahle M, Fukalova J, Kalendova A, Yildirim S, Dzijak R, Dingova-Krasna H, Hozak P (2010) Actin complexes in the cell nucleus: new stones in an old field. *Histochem Cell Biol* **133**(6)**:** 607-626

Chen D, Huang S (2001) Nucleolar components involved in ribosome biogenesis cycle between the nucleolus and nucleoplasm in interphase cells. *J Cell Biol* **153**(1)**:** 169-176

Cho YD, Yoon WJ, Woo KM, Baek JH, Lee G, Cho JY, Ryoo HM (2009) Molecular regulation of matrix extracellular phosphoglycoprotein expression by bone morphogenetic protein-2. *J Biol Chem* **284**(37)**:** 25230-25240

Choi JY, Pratap J, Javed A, Zaidi SK, Xing L, Balint E, Dalamangas S, Boyce B, van Wijnen AJ, Lian JB, Stein JL, Jones SN, Stein GS (2001) Subnuclear targeting of Runx/Cbfa/AML factors is essential for tissue-specific differentiation during embryonic development. *Proc Natl Acad Sci U S A* **98**(15)**:** 8650-8655

Coffman JA (2003) Runx transcription factors and the developmental balance between cell proliferation and differentiation. *Cell Biol Int* **27**(4)**:** 315-324

Cohen MM, Jr. (2009) Perspectives on RUNX genes: an update. *Am J Med Genet A* **149A**(12)**:** 2629-2646

Cunningham CC, Gorlin JB, Kwiatkowski DJ, Hartwig JH, Janmey PA, Byers HR, Stossel TP (1992) Actin-binding protein requirement for cortical stability and efficient locomotion. *Science* **255**(5042)**:** 325-327

Dai H, Xue Y, Pan J, Wu Y, Wang Y, Shen J, Zhang J (2007) Two novel translocations disrupt the RUNX1 gene in acute myeloid leukemia. *Cancer Genet Cytogenet* **177**(2)**:** 120-124

Dallas SL, Bonewald LF (2010) Dynamics of the transition from osteoblast to osteocyte. *Ann N Y Acad Sci* **1192:** 437-443

de Bruijn MF, Speck NA (2004) Core-binding factors in hematopoiesis and immune function. *Oncogene* **23**(24)**:** 4238-4248

de Lanerolle P, Johnson T, Hofmann WA (2005) Actin and myosin I in the nucleus: what next? *Nat Struct Mol Biol* **12**(9)**:** 742-746

Del Fattore A, Capannolo M, Rucci N (2010) Bone and bone marrow: the same organ. *Arch Biochem Biophys* **503**(1)**:** 28-34

Deng W, Roberts SG (2007) TFIIB and the regulation of transcription by RNA polymerase II. *Chromosoma* **116**(5)**:** 417-429

Dingova H, Fukalova J, Maninova M, Philimonenko VV, Hozak P (2009) Ultrastructural localization of actin and actin-binding proteins in the nucleus. *Histochem Cell Biol* **131**(3)**:** 425-434

Dobbs MB, Boehm S, Grange DK, Gurnett CA (2008) Case report: Congenital knee dislocation in a patient with larsen syndrome and a novel filamin B mutation. *Clin Orthop Relat Res* **466**(6)**:** 1503-1509

Dorozhkin SV, Epple M (2002) Biological and medical significance of calcium phosphates. *Angew Chem Int Ed Engl* **41**(17)**:** 3130-3146

Drissi H, Pouliot A, Koolloos C, Stein JL, Lian JB, Stein GS, van Wijnen AJ (2002) 1,25-(OH)2-vitamin D3 suppresses the bone-related Runx2/Cbfa1 gene promoter. *Exp Cell Res* **274**(2)**:** 323-333

Ducy P, Karsenty G (1995) Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. *Mol Cell Biol* **15**(4)**:** 1858-1869

Ducy P, Karsenty G (1998) Genetic control of cell differentiation in the skeleton. *Curr Opin Cell Biol* **10**(5)**:** 614-619

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G (1997) Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* **89**(5)**:** 747-754

Eferl R, Hoebertz A, Schilling AF, Rath M, Karreth F, Kenner L, Amling M, Wagner EF (2004) The Fos-related antigen Fra-1 is an activator of bone matrix formation. *EMBO J* **23**(14)**:** 2789-2799

Emmott E, Hiscox JA (2009) Nucleolar targeting: the hub of the matter. *EMBO Rep* **10**(3)**:** 231-238

Farrington-Rock C, Firestein MH, Bicknell LS, Superti-Furga A, Bacino CA, Cormier-Daire V, Le Merrer M, Baumann C, Roume J, Rump P, Verheij JB, Sweeney E, Rimoin DL, Lachman RS, Robertson SP, Cohn DH, Krakow D (2006) Mutations in two regions of FLNB result in atelosteogenesis I and III. *Hum Mutat* **27**(7)**:** 705-710

Farrington-Rock C, Kirilova V, Dillard-Telm L, Borowsky AD, Chalk S, Rock MJ, Cohn DH, Krakow D (2008) Disruption of the Flnb gene in mice phenocopies the human disease spondylocarpotarsal synostosis syndrome. *Hum Mol Genet* **17**(5)**:** 631-641

Feng Y, Chen MH, Moskowitz IP, Mendonza AM, Vidali L, Nakamura F, Kwiatkowski DJ, Walsh CA (2006) Filamin A (FLNA) is required for cell-cell contact in vascular development and cardiac morphogenesis. *Proc Natl Acad Sci U S A* **103**(52)**:** 19836-19841

Feng Y, Walsh CA (2004) The many faces of filamin: a versatile molecular scaffold for cell motility and signalling. *Nat Cell Biol* **6**(11)**:** 1034-1038

Foley C, Roberts K, Tchrakian N, Morgan T, Fryer A, Robertson SP, Tubridy N (2010) Expansion of the Spectrum of FLNA Mutations Associated with Melnick-Needles Syndrome. *Mol Syndromol* **1**(3)**:** 121-126

Franceschi RT, Iyer BS, Cui Y (1994) Effects of ascorbic acid on collagen matrix formation and osteoblast differentiation in murine MC3T3-E1 cells. *J Bone Miner Res* **9**(6)**:** 843-854

Franceschi RT, Xiao G (2003) Regulation of the osteoblast-specific transcription factor, Runx2: responsiveness to multiple signal transduction pathways. *J Cell Biochem* **88**(3)**:** 446-454

Galkin VE, Orlova, A., Fattoum, A., Walsh, M. P. and Egelman, E. H. (2006) The CH-domain of calponin does not determine the modes of calponin binding to F-actin. . *J Mol Biol* **359:** 478-485.

Geoffroy V, Ducy P, Karsenty G (1995) A PEBP2 alpha/AML-1-related factor increases osteocalcin promoter activity through its binding to an osteoblast-specific cis-acting element. *J Biol Chem* **270**(52)**:** 30973-30979

Gettemans J, Van Impe K, Delanote V, Hubert T, Vandekerckhove J, De Corte V (2005) Nuclear actin-binding proteins as modulators of gene transcription. *Traffic* **6**(10)**:** 847-857

Gollner H, Dani C, Phillips B, Philipsen S, Suske G (2001) Impaired ossification in mice lacking the transcription factor Sp3. *Mech Dev* **106**(1-2)**:** 77-83

Gordeladze JO, Reseland JE, Duroux-Richard I, Apparailly F, Jorgensen C (2009) From stem cells to bone: phenotype acquisition, stabilization, and tissue engineering in animal models. *ILAR J* **51**(1)**:** 42-61

Grummt I (2003) Life on a planet of its own: regulation of RNA polymerase I transcription in the nucleolus. *Genes Dev* **17**(14)**:** 1691-1702

Hacia JG, Brody LC, Collins FS (1998) Applications of DNA chips for genomic analysis. *Mol Psychiatry* **3**(6)**:** 483-492

Hadjiolova KV, Hadjiolov AA, Bachellerie JP (1995) Actinomycin D stimulates the transcription of rRNA minigenes transfected into mouse cells. Implications for the in vivo hypersensitivity of rRNA gene transcription. *Eur J Biochem* **228**(3)**:** 605-615

Harada S, Matsumoto T, Ogata E (1991) Role of ascorbic acid in the regulation of proliferation in osteoblast-like MC3T3-E1 cells. *J Bone Miner Res* **6**(9)**:** 903-908

Hart AW, Morgan JE, Schneider J, West K, McKie L, Bhattacharya S, Jackson IJ, Cross SH (2006) Cardiac malformations and midline skeletal defects in mice lacking filamin A. *Hum Mol Genet* **15**(16)**:** 2457-2467

Hartmann C (2009) Transcriptional networks controlling skeletal development. *Curr Opin Genet Dev* **19**(5)**:** 437-443

Hernandez-Verdun D, Louvet E (2004) [The nucleolus: structure, functions, amd associated diseases]. *Med Sci (Paris)* **20**(1)**:** 37-44

Heuze ML, Lamsoul I, Baldassarre M, Lad Y, Leveque S, Razinia Z, Moog-Lutz C, Calderwood DA, Lutz PG (2008) ASB2 targets filamins A and B to proteasomal degradation. *Blood* **112**(13)**:** 5130-5140

Holmes KC, Popp D, Gebhard W, Kabsch W (1990) Atomic model of the actin filament. *Nature* **347**(6288)**:** 44-49

Hong D, Chen HX, Yu HQ, Liang Y, Wang C, Lian QQ, Deng HT, Ge RS (2010) Morphological and proteomic analysis of early stage of osteoblast differentiation in osteoblastic progenitor cells. *Exp Cell Res* **316**(14)**:** 2291-2300

Hozak P, Cook PR, Schofer C, Mosgoller W, Wachtler F (1994) Site of transcription of ribosomal RNA and intranucleolar structure in HeLa cells. *J Cell Sci* **107 (Pt 2):** 639-648

Huang da W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* **4**(1)**:** 44-57

Huang G, Shigesada K, Ito K, Wee HJ, Yokomizo T, Ito Y (2001) Dimerization with PEBP2beta protects RUNX1/AML1 from ubiquitin-proteasome-mediated degradation. *Embo J* **20**(4)**:** 723-733

Ito Y (2004) Oncogenic potential of the RUNX gene family: 'overview'. *Oncogene* **23**(24)**:** 4198-4208

Ito Y (2008) RUNX Genes in Development and Cancer: Regulation of Viral Gene Expression and the Discovery of RUNX Family Genes. *Adv Cancer Res* **99C:** 33-76

Ito Y, Miyazono K (2003) RUNX transcription factors as key targets of TGF-beta superfamily signaling. *Curr Opin Genet Dev* **13**(1)**:** 43-47

Janmey PA (1998) The cytoskeleton and cell signaling: component localization and mechanical coupling. *Physiol Rev* **78**(3)**:** 763-781

Jarrous N, Wolenski JS, Wesolowski D, Lee C, Altman S (1999) Localization in the nucleolus and coiled bodies of protein subunits of the ribonucleoprotein ribonuclease P. *J Cell Biol* **146**(3)**:** 559-572

Javed A, Guo B, Hiebert S, Choi JY, Green J, Zhao SC, Osborne MA, Stifani S, Stein JL, Lian JB, van Wijnen AJ, Stein GS (2000) Groucho/TLE/R-esp proteins associate

with the nuclear matrix and repress RUNX (CBF(alpha)/AML/PEBP2(alpha)) dependent activation of tissue-specific gene transcription. *J Cell Sci* **113 (Pt 12):** 2221-2231

Jayapal M, Melendez AJ (2006) DNA microarray technology for target identification and validation. *Clin Exp Pharmacol Physiol* **33**(5-6)**:** 496-503

Jensen ED, Gopalakrishnan R, Westendorf JJ (2010) Regulation of gene expression in osteoblasts. *Biofactors* **36**(1)**:** 25-32

Junera HR, Masson C, Geraud G, Hernandez-Verdun D (1995) The three-dimensional organization of ribosomal genes and the architecture of the nucleoli vary with G1, S and G2 phases. *J Cell Sci* **108 (Pt 11):** 3427-3441

Kamachi Y, Ogawa E, Asano M, Ishida S, Murakami Y, Satake M, Ito Y, Shigesada K (1990) Purification of a mouse nuclear factor that binds to both the A and B cores of the polyomavirus enhancer. *J Virol* **64**(10)**:** 4808-4819

Kanno Y, Kanno T, Sakakura C, Bae SC, Ito Y (1998) Cytoplasmic sequestration of the polyomavirus enhancer binding protein 2 (PEBP2)/core binding factor alpha (CBFalpha) subunit by the leukemia-related PEBP2/CBFbeta-SMMHC fusion protein inhibits PEBP2/CBF-mediated transactivation. *Mol Cell Biol* **18**(7)**:** 4252-4261

Karsenty G (2001) Genetic control of skeletal development. *Novartis Found Symp* **232:** 6-17; discussion 17-22

Kartsogiannis V, Ng KW (2004) Cell lines and primary cell cultures in the study of bone cell biology. *Mol Cell Endocrinol* **228**(1-2)**:** 79-102

Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T, Rosen V, Wozney JM, Fujisawa-Sehara A, Suda T (1994) Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. *J Cell Biol* **127**(6 Pt 1)**:** 1755-1766

Khaitlina SY (2001) Functional specificity of actin isoforms. *Int Rev Cytol* **202:** 35-98

Kim EJ, Park JS, Um SJ (2007) Filamin A negatively regulates the transcriptional activity of p73alpha in the cytoplasm. *Biochem Biophys Res Commun* **362**(4)**:** 1101-1106

Kim HJ, Kim JH, Bae SC, Choi JY, Kim HJ, Ryoo HM (2003) The protein kinase C pathway plays a central role in the fibroblast growth factor-stimulated expression and transactivation activity of Runx2. *J Biol Chem* **278**(1)**:** 319-326

Kim HJ, Nam SH, Park HS, Ryoo HM, Kim SY, Cho TJ, Kim SG, Bae SC, Kim IS, Stein JL, van Wijnen AJ, Stein GS, Lian JB, Choi JY (2006) Four novel RUNX2 mutations including a splice donor site result in the cleidocranial dysplasia phenotype. *J Cell Physiol* **207**(1)**:** 114-122

Kimura A, Inose H, Yano F, Fujita K, Ikeda T, Sato S, Iwasaki M, Jinno T, Ae K, Fukumoto S, Takeuchi Y, Itoh H, Imamura T, Kawaguchi H, Chung UI, Martin JF, Iseki S, Shinomiya K, Takeda S (2010) Runx1 and Runx2 cooperate during sternal morphogenesis. *Development* **137**(7)**:** 1159-1167

Kobayashi Y, Udagawa N, Takahashi N (2009) Action of RANKL and OPG for osteoclastogenesis. *Crit Rev Eukaryot Gene Expr* **19**(1)**:** 61-72

Komori T (2003) Requisite roles of Runx2 and Cbfb in skeletal development. *J Bone Miner Metab* **21**(4)**:** 193-197

Komori T (2006) Regulation of osteoblast differentiation by transcription factors. *J Cell Biochem* **99**(5)**:** 1233-1239

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T (1997) Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* **89**(5)**:** 755-764

Krakow D, Robertson SP, King LM, Morgan T, Sebald ET, Bertolotto C, Wachsmann-Hogiu S, Acuna D, Shapiro SS, Takafuta T, Aftimos S, Kim CA, Firth H, Steiner CE, Cormier-Daire V, Superti-Furga A, Bonafe L, Graham JM, Jr., Grix A, Bacino CA, Allanson J, Bialer MG, Lachman RS, Rimoin DL, Cohn DH (2004) Mutations in the gene encoding filamin B disrupt vertebral segmentation, joint formation and skeletogenesis. *Nat Genet* **36**(4)**:** 405-410

Kubota S, Siomi H, Satoh T, Endo S, Maki M, Hatanaka M (1989) Functional similarity of HIV-I rev and HTLV-I rex proteins: identification of a new nucleolar-targeting signal in rev protein. *Biochem Biophys Res Commun* **162**(3)**:** 963-970

Kukalev A, Nord Y, Palmberg C, Bergman T, Percipalle P (2005) Actin and hnRNP U cooperate for productive transcription by RNA polymerase II. *Nat Struct Mol Biol* **12**(3)**:** 238-244

Kuo MH, Allis CD (1999) In vivo cross-linking and immunoprecipitation for studying dynamic Protein:DNA associations in a chromatin environment. *Methods* **19**(3)**:** 425-433

Kwak KB, Chung SS, Kim OM, Kang MS, Ha DB, Chung CH (1993) Increase in the level of m-calpain correlates with the elevated cleavage of filamin during myogenic differentiation of embryonic muscle cells. *Biochim Biophys Acta* **1175**(3)**:** 243-249

Larijani B, Poccia DL (2009) Nuclear envelope formation: mind the gaps. *Annu Rev Biophys* **38:** 107-124

Lee B, Thirunavukkarasu K, Zhou L, Pastore L, Baldini A, Hecht J, Geoffroy V, Ducy P, Karsenty G (1997) Missense mutations abolishing DNA binding of the osteoblast-specific transcription factor OSF2/CBFA1 in cleidocranial dysplasia. *Nat Genet* **16**(3)**:** 307-310

Lee MH, Kim YJ, Yoon WJ, Kim JI, Kim BG, Hwang YS, Wozney JM, Chi XZ, Bae SC, Choi KY, Cho JY, Choi JY, Ryoo HM (2005) Dlx5 specifically regulates Runx2 type II expression by binding to homeodomain-response elements in the Runx2 distal promoter. *J Biol Chem* **280**(42)**:** 35579-35587

Levanon D, Bettoun D, Harris-Cerruti C, Woolf E, Negreanu V, Eilam R, Bernstein Y, Goldenberg D, Xiao C, Fliegauf M, Kremer E, Otto F, Brenner O, Lev-Tov A, Groner Y (2002) The Runx3 transcription factor regulates development and survival of TrkC dorsal root ganglia neurons. *EMBO J* **21**(13)**:** 3454-3463

Li Q, Lau A, Morris TJ, Guo L, Fordyce CB, Stanley EF (2004) A syntaxin 1, Galpha(o), and N-type calcium channel complex at a presynaptic nerve terminal: analysis by quantitative immunocolocalization. *J Neurosci* **24**(16)**:** 4070-4081

Li QL, Ito K, Sakakura C, Fukamachi H, Inoue K, Chi XZ, Lee KY, Nomura S, Lee CW, Han SB, Kim HM, Kim WJ, Yamamoto H, Yamashita N, Yano T, Ikeda T, Itohara S, Inazawa J, Abe T, Hagiwara A, Yamagishi H, Ooe A, Kaneda A, Sugimura T, Ushijima T, Bae SC, Ito Y (2002) Causal relationship between the loss of RUNX3 expression and gastric cancer. *Cell* **109**(1)**:** 113-124

Lian JB, Stein GS (2003) Runx2/Cbfa1: a multifunctional regulator of bone formation. *Curr Pharm Des* **9**(32)**:** 2677-2685

Lian JB, Stein GS, Javed A, van Wijnen AJ, Stein JL, Montecino M, Hassan MQ, Gaur T, Lengner CJ, Young DW (2006) Networks and hubs for the transcriptional control of osteoblastogenesis. *Rev Endocr Metab Disord* **7**(1-2)**:** 1-16

Lin X, Duan X, Liang YY, Su Y, Wrighton KH, Long J, Hu M, Davis CM, Wang J, Brunicardi FC, Shi Y, Chen YG, Meng A, Feng XH (2006) PPM1A functions as a Smad phosphatase to terminate TGFbeta signaling. *Cell* **125**(5)**:** 915-928

Lindstrom MS, Klangby U, Inoue R, Pisa P, Wiman KG, Asker CE (2000) Immunolocalization of human p14(ARF) to the granular component of the interphase nucleolus. *Exp Cell Res* **256**(2)**:** 400-410

Liu W, Toyosawa S, Furuichi T, Kanatani N, Yoshida C, Liu Y, Himeno M, Narai S, Yamaguchi A, Komori T (2001) Overexpression of Cbfa1 in osteoblasts inhibits osteoblast maturation and causes osteopenia with multiple fractures. *J Cell Biol* **155**(1)**:** 157-166

Loy CJ, Sim KS, Yong EL (2003) Filamin-A fragment localizes to the nucleus to regulate androgen receptor and coactivator functions. *Proc Natl Acad Sci U S A* **100**(8)**:** 4562-4567

Lu J, Lian G, Lenkinski R, De Grand A, Vaid RR, Bryce T, Stasenko M, Boskey A, Walsh C, Sheen V (2007) Filamin B mutations cause chondrocyte defects in skeletal development. *Hum Mol Genet* **16**(14)**:** 1661-1675

Lu J, Sheen V (2005) Periventricular heterotopia. *Epilepsy Behav* **7**(2)**:** 143-149

Lutterbach B, Westendorf JJ, Linggi B, Isaac S, Seto E, Hiebert SW (2000) A mechanism of repression by acute myeloid leukemia-1, the target of multiple chromosomal translocations in acute leukemia. *J Biol Chem* **275**(1)**:** 651-656

Marino-Enriquez A, Lapunzina P, Robertson SP, Rodriguez JI (2007) Otopalatodigital syndrome type 2 in two siblings with a novel filamin A 629G>T mutation: clinical, pathological, and molecular findings. *Am J Med Genet A* **143A**(10)**:** 1120-1125

Maruyama Z, Komori T (2007) [Function of Osterix in osteoblasts]. *Nippon Rinsho* **65 Suppl 9:** 81-83

Matsubara T, Kida K, Yamaguchi A, Hata K, Ichida F, Meguro H, Aburatani H, Nishimura R, Yoneda T (2008) BMP2 regulates Osterix through Msx2 and Runx2 during osteoblast differentiation. *J Biol Chem* **283**(43)**:** 29119-29125

Mattot V, Raes MB, Henriet P, Eeckhout Y, Stehelin D, Vandenbunder B, Desbiens X (1995) Expression of interstitial collagenase is restricted to skeletal tissue during mouse embryogenesis. *J Cell Sci* **108 (Pt 2):** 529-535

McLarren KW, Lo R, Grbavec D, Thirunavukkarasu K, Karsenty G, Stifani S (2000) The mammalian basic helix loop helix protein HES-1 binds to and modulates the transactivating function of the runt-related factor Cbfa1. *J Biol Chem* **275**(1)**:** 530-538

McStay B, Grummt I (2008) The epigenetics of rRNA genes: from molecular to chromosome biology. *Annu Rev Cell Dev Biol* **24:** 131-157

Meder VS, Boeglin M, de Murcia G, Schreiber V (2005) PARP-1 and PARP-2 interact with nucleophosmin/B23 and accumulate in transcriptionally active nucleoli. *J Cell Sci* **118**(Pt 1)**:** 211-222

Mendoza-Villanueva D, Deng W, Lopez-Camacho C, Shore P (2010) The Runx transcriptional co-activator, CBFbeta, is essential for invasion of breast cancer cells. *Mol Cancer* **9:** 171

Miralles F, Posern G, Zaromytidou AI, Treisman R (2003) Actin dynamics control SRF activity by regulation of its coactivator MAL. *Cell* **113**(3)**:** 329-342

Mizutani A, Sugiyama I, Kuno E, Matsunaga S, Tsukagoshi N (2001) Expression of matrix metalloproteinases during ascorbate-induced differentiation of osteoblastic MC3T3-E1 cells. *J Bone Miner Res* **16**(11)**:** 2043-2049

Moss T, Langlois F, Gagnon-Kugler T, Stefanovsky V (2007) A housekeeper with power of attorney: the rRNA genes in ribosome biogenesis. *Cell Mol Life Sci* **64**(1)**:** 29-49

Muller C, Bremer A, Schreiber S, Eichwald S, Calkhoven CF (2010) Nucleolar retention of a translational C/EBPalpha isoform stimulates rDNA transcription and cell size. *EMBO J* **29**(5)**:** 897-909

Mundlos S (1999) Cleidocranial dysplasia: clinical and molecular genetics. *J Med Genet* **36**(3)**:** 177-182

Nagata T, Gupta V, Sorce D, Kim WY, Sali A, Chait BT, Shigesada K, Ito Y, Werner MH (1999) Immunoglobulin motif DNA recognition and heterodimerization of the PEBP2/CBF Runt domain. *Nat Struct Biol* **6**(7)**:** 615-619

Nakashima K, de Crombrugghe B (2003) Transcriptional mechanisms in osteoblast differentiation and bone formation. *Trends Genet* **19**(8)**:** 458-466

Napierala D, Garcia-Rojas X, Sam K, Wakui K, Chen C, Mendoza-Londono R, Zhou G, Zheng Q, Lee B (2005) Mutations and promoter SNPs in RUNX2, a transcriptional regulator of bone formation. *Mol Genet Metab* **86**(1-2)**:** 257-268

Nimmo R, Woollard A (2007) Worming out the biology of Runx. *Dev Biol*

Obrdlik A, Kukalev A, Percipalle P (2007) The function of actin in gene transcription. *Histol Histopathol* **22**(9)**:** 1051-1055

Oda T, Maeda Y (2010) Multiple Conformations of F-actin. *Structure* **18**(7)**:** 761-767

Ogawa E, Inuzuka M, Maruyama M, Satake M, Naito-Fujimoto M, Ito Y, Shigesada K (1993) Molecular cloning and characterization of PEBP2 beta, the heterodimeric partner of a novel Drosophila runt-related DNA binding protein PEBP2 alpha. *Virology* **194**(1)**:** 314-331

Okuda T, van Deursen J, Hiebert SW, Grosveld G, Downing JR (1996) AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell* **84**(2)**:** 321-330

Olave IA, Reck-Peterson SL, Crabtree GR (2002) Nuclear actin and actin-related proteins in chromatin remodeling. *Annu Rev Biochem* **71:** 755-781

Olson MO, Dundr M, Szebeni A (2000) The nucleolus: an old factory with unexpected capabilities. *Trends Cell Biol* **10**(5)**:** 189-196

Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB, Owen MJ (1997) Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell* **89**(5)**:** 765-771

Ozanne DM, Brady ME, Cook S, Gaughan L, Neal DE, Robson CN (2000) Androgen receptor nuclear translocation is facilitated by the f-actin cross-linking protein filamin. *Mol Endocrinol* **14**(10)**:** 1618-1626

Pederson T (1998) The plurifunctional nucleolus. *Nucleic Acids Res* **26**(17)**:** 3871-3876

Pelletier N, Champagne N, Stifani S, Yang XJ (2002) MOZ and MORF histone acetyltransferases interact with the Runt-domain transcription factor Runx2. *Oncogene* **21**(17)**:** 2729-2740

Perry RP, Kelley DE (1970) Inhibition of RNA synthesis by actinomycin D: characteristic dose-response of different RNA species. *J Cell Physiol* **76**(2)**:** 127-139

Philimonenko VV, Zhao J, Iben S, Dingova H, Kysela K, Kahle M, Zentgraf H, Hofmann WA, de Lanerolle P, Hozak P, Grummt I (2004) Nuclear actin and myosin I are required for RNA polymerase I transcription. *Nat Cell Biol* **6**(12)**:** 1165-1172

Phimphilai M, Zhao Z, Boules H, Roca H, Franceschi RT (2006) BMP signaling is required for RUNX2-dependent induction of the osteoblast phenotype. *J Bone Miner Res* **21**(4)**:** 637-646

Popowicz GM, Schleicher, M., Noegel, A. A. and Holak, T. A. (2006) Filamins: promiscuous organizers of the cytoskeleton. *Trends Biochem Sci* **31:** 411-419.

Prince M, Banerjee C, Javed A, Green J, Lian JB, Stein GS, Bodine PV, Komm BS (2001) Expression and regulation of Runx2/Cbfa1 and osteoblast phenotypic markers during the growth and differentiation of human osteoblasts. *J Cell Biochem* **80**(3)**:** 424-440

Provenzano M, Mocellin S (2007) Complementary techniques: validation of gene expression data by quantitative real time PCR. *Adv Exp Med Biol* **593:** 66-73

Puig-Kroger A, Corbi A (2006) RUNX3: a new player in myeloid gene expression and immune response. *J Cell Biochem* **98**(4)**:** 744-756

Puvion-Dutilleul F, Puvion E, Bachellerie JP (1997) Early stages of pre-rRNA formation within the nucleolar ultrastructure of mouse cells studied by in situ hybridization with a 5'ETS leader probe. *Chromosoma* **105**(7-8)**:** 496-505

Quack I, Vonderstrass B, Stock M, Aylsworth AS, Becker A, Brueton L, Lee PJ, Majewski F, Mulliken JB, Suri M, Zenker M, Mundlos S, Otto F (1999) Mutation analysis of core binding factor A1 in patients with cleidocranial dysplasia. *Am J Hum Genet* **65**(5)**:** 1268-1278

Rennert J, Coffman JA, Mushegian AR, Robertson AJ (2003) The evolution of Runx genes I. A comparative study of sequences from phylogenetically diverse model organisms. *BMC Evol Biol* **3:** 4

Robertson SP (2005) Filamin A: phenotypic diversity. *Curr Opin Genet Dev* **15**(3)**:** 301-307

Robertson SP (2007) Otopalatodigital syndrome spectrum disorders: otopalatodigital syndrome types 1 and 2, frontometaphyseal dysplasia and Melnick-Needles syndrome. *Eur J Hum Genet* **15**(1)**:** 3-9

Robertson SP, Twigg SR, Sutherland-Smith AJ, Biancalana V, Gorlin RJ, Horn D, Kenwrick SJ, Kim CA, Morava E, Newbury-Ecob R, Orstavik KH, Quarrell OW, Schwartz CE, Shears DJ, Suri M, Kendrick-Jones J, Wilkie AO (2003) Localized mutations in the gene encoding the cytoskeletal protein filamin A cause diverse malformations in humans. *Nat Genet* **33**(4)**:** 487-491

Russell J, Zomerdijk JC (2006) The RNA polymerase I transcription machinery. *Biochem Soc Symp*(73)**:** 203-216

Safran M, Dalah I, Alexander J, Rosen N, Iny Stein T, Shmoish M, Nativ N, Bahir I, Doniger T, Krug H, Sirota-Madi A, Olender T, Golan Y, Stelzer G, Harel A, Lancet D (2010) GeneCards Version 3: the human gene integrator. *Database (Oxford)* **2010:** baq020

Samee N, Geoffroy V, Marty C, Schiltz C, Vieux-Rochas M, Levi G, de Vernejoul MC (2008) Dlx5, a positive regulator of osteoblastogenesis, is essential for osteoblast-osteoclast coupling. *Am J Pathol* **173**(3)**:** 773-780

Sasaki A, Masuda Y, Ohta Y, Ikeda K, Watanabe K (2001) Filamin associates with Smads and regulates transforming growth factor-beta signaling. *J Biol Chem* **276**(21)**:** 17871-17877

Sauman I, Berry SJ (1994) An actin infrastructure is associated with eukaryotic chromosomes: structural and functional significance. *Eur J Cell Biol* **64**(2)**:** 348-356

Scheer U, Benavente R (1990) Functional and dynamic aspects of the mammalian nucleolus. *Bioessays* **12**(1)**:** 14-21

Schroeder TM, Jensen ED, Westendorf JJ (2005) Runx2: a master organizer of gene transcription in developing and maturing osteoblasts. *Birth Defects Res C Embryo Today* **75**(3)**:** 213-225

Schwarzacher HG, Mosgoeller W (2000) Ribosome biogenesis in man: current views on nucleolar structures and function. *Cytogenet Cell Genet* **91**(1-4)**:** 243-252

Scott M, Boisvert FM, Vieyra D, Johnston RN, Bazett-Jones DP, Riabowol K (2001) UV induces nucleolar translocation of ING1 through two distinct nucleolar targeting sequences. *Nucleic Acids Res* **29**(10)**:** 2052-2058

Selvamurugan N, Kwok S, Partridge NC (2004) Smad3 interacts with JunB and Cbfa1/Runx2 for transforming growth factor-beta1-stimulated collagenase-3 expression in human breast cancer cells. *J Biol Chem* **279**(26)**:** 27764-27773

Shav-Tal Y, Blechman J, Darzacq X, Montagna C, Dye BT, Patton JG, Singer RH, Zipori D (2005) Dynamic sorting of nuclear components into distinct nucleolar caps during transcriptional inhibition. *Mol Biol Cell* **16**(5)**:** 2395-2413

Sheen VL, Feng Y, Graham D, Takafuta T, Shapiro SS, Walsh CA (2002) Filamin A and Filamin B are co-expressed within neurons during periods of neuronal migration and can physically interact. *Hum Mol Genet* **11**(23)**:** 2845-2854

Shirakabe K, Terasawa K, Miyama K, Shibuya H, Nishida E (2001) Regulation of the activity of the transcription factor Runx2 by two homeobox proteins, Msx2 and Dlx5. *Genes Cells* **6**(10)**:** 851-856

Shum L, Nuckolls G (2002) The life cycle of chondrocytes in the developing skeleton. *Arthritis Res* **4**(2)**:** 94-106

Sierra J, Villagra A, Paredes R, Cruzat F, Gutierrez S, Javed A, Arriagada G, Olate J, Imschenetzky M, Van Wijnen AJ, Lian JB, Stein GS, Stein JL, Montecino M (2003) Regulation of the bone-specific osteocalcin gene by p300 requires Runx2/Cbfa1 and the vitamin D3 receptor but not p300 intrinsic histone acetyltransferase activity. *Mol Cell Biol* **23**(9)**:** 3339-3351

Sirri V, Urcuqui-Inchima S, Roussel P, Hernandez-Verdun D (2008) Nucleolus: the fascinating nuclear body. *Histochem Cell Biol* **129**(1)**:** 13-31

Skarp KP, Vartiainen MK (2010) Actin on DNA-an ancient and dynamic relationship. *Cytoskeleton (Hoboken)* **67**(8)**:** 487-495

Smetana K, Busch R, Chan PK, Smetana K, Jr., Busch H (2001) Immunocytochemical localization of nucleophosmin and RH-II/Gu protein in nucleoli of HeLa cells after treatment with actinomycin D. *Acta Histochem* **103**(3)**:** 325-333

Song CZ, Hanada K, Yano K, Maeda Y, Yamamoto K, Muramatsu M (1994) High conservation of subunit composition of RNA polymerase I(A) between yeast and mouse and the molecular cloning of mouse RNA polymerase I 40-kDa subunit RPA40. *J Biol Chem* **269**(43)**:** 26976-26981

Stein GS, Lian JB, van Wijnen AJ, Stein JL, Montecino M, Javed A, Zaidi SK, Young DW, Choi JY, Pockwinse SM (2004) Runx2 control of organization, assembly and activity of the regulatory machinery for skeletal gene expression. *Oncogene* **23**(24)**:** 4315-4329

Stein GS, van Wijnen AJ, Stein JL, Lian JB, Montecino M, Zaidi K, Javed A (2000) Subnuclear organization and trafficking of regulatory proteins: implications for biological control and cancer. *J Cell Biochem Suppl* **Suppl 35:** 84-92

Stock M, Otto F (2005) Control of RUNX2 isoform expression: the role of promoters and enhancers. *J Cell Biochem* **95**(3)**:** 506-517

Stossel TP, Condeelis J, Cooley L, Hartwig JH, Noegel A, Schleicher M, Shapiro SS (2001) Filamins as integrators of cell mechanics and signalling. *Nat Rev Mol Cell Biol* **2**(2)**:** 138-145

Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ (1999) Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. *Endocr Rev* **20**(3)**:** 345-357

Sun Y, Almomani R, Aten E, Celli J, van der Heijden J, Venselaar H, Robertson SP, Baroncini A, Franco B, Basel-Vanagaite L, Horii E, Drut R, Ariyurek Y, den Dunnen JT, Breuning MH (2010) Terminal osseous dysplasia is caused by a single recurrent mutation in the FLNA gene. *Am J Hum Genet* **87**(1)**:** 146-153

Takasu H (2008) Anti-osteoclastogenic action of active vitamin D. *Nutr Rev* **66**(10 Suppl 2)**:** S113-115

Tan S, Guschin D, Davalos A, Lee YL, Snowden AW, Jouvenot Y, Zhang HS, Howes K, McNamara AR, Lai A, Ullman C, Reynolds L, Moore M, Isalan M, Berg LP, Campos B, Qi H, Spratt SK, Case CC, Pabo CO, Campisi J, Gregory PD (2003) Zinc-finger protein-targeted gene regulation: genomewide single-gene specificity. *Proc Natl Acad Sci U S A* **100**(21)**:** 11997-12002

Thirunavukkarasu K, Mahajan M, McLarren KW, Stifani S, Karsenty G (1998) Two domains unique to osteoblast-specific transcription factor Osf2/Cbfa1 contribute to its transactivation function and its inability to heterodimerize with Cbfbeta. *Mol Cell Biol* **18**(7)**:** 4197-4208

Thomas P, Bossan A, Lacour JP, Chanalet S, Ortonne JP, Chatel M (1996) Ehlers-Danlos syndrome with subependymal periventricular heterotopias. *Neurology* **46**(4)**:** 1165-1167

Tollervey D, Hurt EC (1990) The role of small nucleolar ribonucleoproteins in ribosome synthesis. *Mol Biol Rep* **14**(2-3)**:** 103-106

Uribe R, Jay D (2009) A review of actin binding proteins: new perspectives. *Mol Biol Rep* **36**(1)**:** 121-125

van der Flier A, Kuikman I, Kramer D, Geerts D, Kreft M, Takafuta T, Shapiro SS, Sonnenberg A (2002) Different splice variants of filamin-B affect myogenesis, subcellular distribution, and determine binding to integrin [beta] subunits. *J Cell Biol* **156**(2)**:** 361-376

van Wijnen AJ, Stein GS, Gergen JP, Groner Y, Hiebert SW, Ito Y, Liu P, Neil JC, Ohki M, Speck N (2004) Nomenclature for Runt-related (RUNX) proteins. *Oncogene* **23**(24)**:** 4209-4210

Vandelaer M, Thiry M, Goessens G (1996) Isolation of nucleoli from ELT cells: a quick new method that preserves morphological integrity and high transcriptional activity. *Exp Cell Res* **228**(1)**:** 125-131

Vega RB, Matsuda K, Oh J, Barbosa AC, Yang X, Meadows E, McAnally J, Pomajzl C, Shelton JM, Richardson JA, Karsenty G, Olson EN (2004) Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. *Cell* **119**(4)**:** 555-566

Visa N, Percipalle P (2010) Nuclear functions of actin. *Cold Spring Harb Perspect Biol* **2**(4)**:** a000620

Wang Q, Stacy T, Binder M, Marin-Padilla M, Sharpe AH, Speck NA (1996) Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proc Natl Acad Sci U S A* **93**(8)**:** 3444-3449

Wang R, Brattain MG (2007) The maximal size of protein to diffuse through the nuclear pore is larger than 60kDa. *FEBS Lett* **581**(17)**:** 3164-3170

Wang Y, Kreisberg JI, Bedolla RG, Mikhailova M, deVere White RW, Ghosh PM (2007) A 90 kDa fragment of filamin A promotes Casodex-induced growth inhibition in Casodex-resistant androgen receptor positive C4-2 prostate cancer cells. *Oncogene* **26**(41)**:** 6061-6070

Wee HJ, Huang G, Shigesada K, Ito Y (2002) Serine phosphorylation of RUNX2 with novel potential functions as negative regulatory mechanisms. *EMBO Rep* **3**(10)**:** 967-974

Wente SR, Rout MP (2010) The nuclear pore complex and nuclear transport. *Cold Spring Harb Perspect Biol* **2**(10)**:** a000562

Westendorf JJ (2006) Transcriptional co-repressors of Runx2. *J Cell Biochem* **98**(1)**:** 54-64

Westendorf JJ, Zaidi SK, Cascino JE, Kahler R, van Wijnen AJ, Lian JB, Yoshida M, Stein GS, Li X (2002) Runx2 (Cbfa1, AML-3) interacts with histone deacetylase 6 and represses the p21(CIP1/WAF1) promoter. *Mol Cell Biol* **22**(22)**:** 7982-7992

Wheeler JC, Shigesada K, Gergen JP, Ito Y (2000) Mechanisms of transcriptional regulation by Runt domain proteins. *Semin Cell Dev Biol* **11**(5)**:** 369-375

Wu TD (2001) Analysing gene expression data from DNA microarrays to identify candidate genes. *J Pathol* **195**(1)**:** 53-65

Xiao G, Jiang D, Gopalakrishnan R, Franceschi RT (2002) Fibroblast growth factor 2 induction of the osteocalcin gene requires MAPK activity and phosphorylation of the osteoblast transcription factor, Cbfa1/Runx2. *J Biol Chem* **277**(39)**:** 36181-36187

Xiao G, Jiang D, Thomas P, Benson MD, Guan K, Karsenty G, Franceschi RT (2000) MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. *J Biol Chem* **275**(6)**:** 4453-4459

Yamagata T, Maki K, Mitani K (2005) Runx1/AML1 in normal and abnormal hematopoiesis. *Int J Hematol* **82**(1)**:** 1-8

Yang X, Karsenty G (2004) ATF4, the osteoblast accumulation of which is determined post-translationally, can induce osteoblast-specific gene expression in non-osteoblastic cells. *J Biol Chem* **279**(45)**:** 47109-47114

Ye J, Zhao J, Hoffmann-Rohrer U, Grummt I (2008) Nuclear myosin I acts in concert with polymeric actin to drive RNA polymerase I transcription. *Genes Dev* **22**(3)**:** 322-330

Yoshida ea (2005) Filamin A-bound PEBP2beta/CBFbeta is retained in the cytoplasm and prevented from functioning as a partner of the Runx1 transcription factor. *Mol Cell Biol* **25**(3)**:** 1003-1012

Young DW, Hassan MQ, Pratap J, Galindo M, Zaidi SK, Lee SH, Yang X, Xie R, Javed A, Underwood JM, Furcinitti P, Imbalzano AN, Penman S, Nickerson JA, Montecino MA, Lian JB, Stein JL, van Wijnen AJ, Stein GS (2007) Mitotic occupancy and lineage-specific transcriptional control of rRNA genes by Runx2. *Nature* **445**(7126)**:** 442-446

Yue J, Wang Q, Lu H, Brenneman M, Fan F, Shen Z (2009) The cytoskeleton protein filamin-A is required for an efficient recombinational DNA double strand break repair. *Cancer Res* **69**(20)**:** 7978-7985

Zaidi SK, Javed A, Choi JY, van Wijnen AJ, Stein JL, Lian JB, Stein GS (2001) A specific targeting signal directs Runx2/Cbfa1 to subnuclear domains and contributes to transactivation of the osteocalcin gene. *J Cell Sci* **114**(Pt 17)**:** 3093-3102

Zaidi SK, Young DW, Montecino M, Lian JB, Stein JL, van Wijnen AJ, Stein GS (2010) Architectural epigenetics: mitotic retention of mammalian transcriptional regulatory information. *Mol Cell Biol* **30**(20)**:** 4758-4766

Zaragoza C, Lopez-Rivera E, Garcia-Rama C, Saura M, Martinez-Ruiz A, Lizarbe TR, Martin-de-Lara F, Lamas S (2006) Cbfa-1 mediates nitric oxide regulation of MMP-13 in osteoblasts. *J Cell Sci* **119**(Pt 9)**:** 1896-1902

Zeng C, van Wijnen AJ, Stein JL, Meyers S, Sun W, Shopland L, Lawrence JB, Penman S, Lian JB, Stein GS, Hiebert SW (1997) Identification of a nuclear matrix targeting signal in the leukemia and bone-related AML/CBF-alpha transcription factors. *Proc Natl Acad Sci U S A* **94**(13)**:** 6746-6751

Zhang L, Lukasik SM, Speck NA, Bushweller JH (2003) Structural and functional characterization of Runx1, CBF beta, and CBF beta-SMMHC. *Blood Cells Mol Dis* **30**(2)**:** 147-156

Zhang YW, Yasui N, Ito K, Huang G, Fujii M, Hanai J, Nogami H, Ochi T, Miyazono K, Ito Y (2000) A RUNX2/PEBP2alpha A/CBFA1 mutation displaying impaired transactivation and Smad interaction in cleidocranial dysplasia. *Proc Natl Acad Sci U S A* **97**(19)**:** 10549-10554

Zheng L, Baek HJ, Karsenty G, Justice MJ (2007) Filamin B represses chondrocyte hypertrophy in a Runx2/Smad3-dependent manner. *J Cell Biol* **178**(1)**:** 121-128

Zhou AX, Hartwig JH, Akyurek LM (2010) Filamins in cell signaling, transcription and organ development. *Trends Cell Biol* **20**(2)**:** 113-123

Zhou X, Tian, F., Sandzen, J. et al. (2007) Filamin B deficiency in mice results in skeletal malformations and impaired microvascular development. *Proc Natl Acad Sci* **104** 3919-3924.